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(21) International Application Number: PCT/US94/12272 (22) International Filing Date: 26 October 1994 (26.10.94) (30) Priority Data: 145,437 29 October 1993 (29.10.93) US (60) Parent Application or Grant (63) Related by Continuation US 145,437 (CIP) Filed on 29 October 1993 (29.10.93) (71) Applicants (for all designated States except US): MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). THE UNIVERSITY OF VIRGINIA PATENTS FOUNDATION [US/US]; Towers Office Building, Suite 1-110, 1224 West Main Street, Charlottesville, VA 22903 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): DOYLE, Michael, P. [US/US]; Route 18, Box 76, Charlottesville, VA 22901 (US). JACOBSON, Mariene, A. [US/US]; 8003 Cooke Road, Elkins Park, PA 19117 (US). DULING, Brian, R. [US/US]; 1920 Meadowbrook Road, Charlottesville, VA		(74) Agent: MERCK & CO., INC.; Patent Dept., 126 East Lincoln Avenue, Rahway, NJ 07065 (US). (81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: HUMAN ADENOSINE RECEPTOR ANTAGONISTS (57) Abstract The invention concerns the use of compounds, identified through the use of recombinant human adenosine receptors A1, A2a, A2b and A3, and functional assays, to specifically modulate the physiologic role of adenosine activation of its various receptors.		

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- 1 -

TITLE OF THE INVENTION

HUMAN ADENOSINE RECEPTOR ANTAGONISTS

RELATED APPLICATIONS

5 This application is a continuation-in-part of USSN
08/145,437, filed on 10/29/93, pending.

GOVERNMENT SUPPORT

10 This invention was made with government support under
RO1HL37942 grant awarded by the National Institutes of Health. The
Government has certain rights in the invention.

BACKGROUND OF THE INVENTION1. FIELD OF THE INVENTION:

15 The present invention concerns the use of compounds
identified as specific modulators of adenosine's physiological actions.
The pharmacology of these compounds is characterized through the use
of cloned human adenosine receptors of the A1, A2a, A2b and A3 class
and their subtypes. Compounds identified as antagonists of the A3
20 adenosine receptor subtype are useful in preventing mast cell
degranulation and are therefore useful in the treatment or prevention of
disease states induced by activation of the A3 receptor and mast cell
activation. These disease states include but are not limited to asthma,
myocardial reperfusion injury, allergic reactions including but not
25 limited to rhinitis, poison ivy induced responses, urticaria, scleroderma,
arthritis, other autoimmune diseases and inflammatory bowel diseases.

2. BACKGROUND:

30 Adenosine is a naturally occurring nucleoside which
exhibits diverse and potent physiological actions in the cardiovascular,
nervous, pulmonary, renal and immune systems. Adenosine has been
demonstrated to terminate supraventricular tachycardia through
blockage of atrioventricular nodal conduction (J.P. DiMarco, et al.,
(1985) J. Am. Col. Cardiol. 6:417-425, A. Munoz, et al., (1984) Eur.
Heart J. 5:735-738). Adenosine is a potent vasodilator except in the
kidney and placenta (R.A. Olsson, (1981) Ann. Rev. Physiol. 43:385-

- 2 -

395). Adenosine produces bronchoconstriction in asthmatics but not in nonasthmatics (Cushly et al., 1984, *Am. Rev. Respir. Dis.* 129:380-384). Adenosine has been implicated as a preventative agent and in treatment of ventricular dysfunction following episodes of regional or global ischemia (M.B. Forman and C.E. Velasco (1991) *Cardiovasc. Drugs and Therapy* 5:901-908) and in cerebral ischemia (M.C. Evans, et al., (1987) *Neurosci. Lett.* 83:287, D.K.J.E., Von Lubitz, et al., (1988) *Stroke* 19:1133).

Dog A1 and A2a adenosine receptors were the first adenosine receptors to be cloned. See F. Libert, et al., (1989) *Science* 244:569-572, C. Maennant, et al., *Biochem. Biophys. Res. Comm.*, (1990) 173:1169-1178, and F. Libert, et al. (1991) *EMBO J.* 10:1677-1682. The rat A1 adenosine receptor was cloned by L.C. Mahan, et al., (1991) *Mol. Pharm.* 40:1-7 and S.M. Reppert, et al., (1991) *Mol. Endocrin.* 5:1037-1048, the rat A2a by Fink et. al., (1992) *Mol. Brain Res.* 14:186-195, and the rat A2b by Stehle et al. (1992) *Mol. Endocrinol.* 6:384-393. Cloning of the rat A3 adenosine receptor was reported by Meyerhof et al., (1991) *FEBS Lett.* 284:155-160 and Zhou et al., (1992) *PNAS USA* 89:7432-7436. Cloning of the sheep A3 adenosine receptor has been reported by Linden et al., (1993) *Mol. Pharm.* 44:524-532. Cloning of the human A1, A2a, A2b and A3 receptors were reported in GB 2264948-A (9/15/93). The human A1 adenosine receptor differs by 18 amino acids from the dog A1 sequence and 16 amino acids from the rat A1 sequence. The human A2a adenosine receptor differs by 28 and 71 amino acids, respectively from the dog and rat A2a sequences. The amino acid sequence for the human A3 receptor is 72% identical with the rat A3 receptor and 85% identical with the sheep A3 receptor sequences.

The actions of adenosine are mediated through G-protein coupled receptors, the A1, A2a, A2b and A3 adenosine receptors. The adenosine receptors were initially classified into A1 and A2 subtypes on the basis of pharmacological criteria and coupling to adenylate cyclase (Van Caulker, D., Muller, M. and Hamprecht, B. (1979) *J. Neurochem.* 33, 999-1003.). Further pharmacological classification of adenosine

- 3 -

receptors prompted subdivision of the A2 class into A2a and A2b subtypes on the basis of high and low affinity, respectively, for adenosine and the agonists NECA and CGS-21680 (Bruns, R.F., Lu, G.H. and Pugsley, T.A. (1986) *Mol. Pharmacol.* **29**, 331-346; Wan, W., Sutherland, G.R. and Geiger, J.D. (1990) *J. Neurochem.* **55**, 1763-1771). The existence of A1, A2a and A2b subtypes has been confirmed by cloning and functional characterization of expressed bovine, canine, rat and human receptors. A fourth subtype, A3, had remained pharmacologically undetected until its recent identification by molecular cloning. The rat A3 sequence, *tgpcr1*, was first cloned from rat testis by Meyerhoff et al. (see above). Subsequently, a cDNA encoding the identical receptor was cloned from striatum and functionally expressed by Zhou et al. (see above). When compared to the other members of the G-protein coupled receptor family, the rat sequence had the highest homology with the adenosine receptors (> 40% overall identity, 58% within the transmembrane regions). When stably expressed in CHO cells, the receptor was found to bind the radioligand ¹²⁵I-APNEA (N⁶-2-(4-amino-3-iodophenyl)ethyladenosine) and when transfected cells were treated with adenosine agonists, cyclic AMP accumulation was inhibited with a potency order of NECA = R-PIA > CGS21680. The rat A3 receptor exhibited a unique pharmacology relative to the A1 and A2 adenosine receptor subtypes and was reported not to bind the xanthine antagonists 1,3-dipropyl-8-phenylxanthine (DPCPX) and xanthine amine congener (XAC). Messenger RNA for the rat A3 adenosine receptor is primarily expressed in the testis.

The sheep homolog of the A3 receptor was cloned from hypophysial pars tuberalis (see Linden et al. above). The sheep receptor is 72% identical to the rat receptor, binds the radioligand ¹²⁵I-ABA and is also coupled to inhibition of cyclic AMP. The agonist affinity order of the sheep receptor is I-ABA > APNEA > NECA ≥ R-PIA >> CPA. The pharmacology of xanthine antagonists was extensively studied and the sheep receptor was found to exhibit high affinity for 8-phenylxanthines with para-acidic substitutions. In contrast to the rat transcript, the expression of the sheep A3 adenosine receptor transcript

- 4 -

is widespread throughout the brain and is most abundant in the lung and spleen. Moderate amounts of transcript are also observed in pineal and testis. Thus, because the published literature provides an inconsistent profile of adenosine A3 receptor pharmacology and tissue distribution,
5 it was not possible to predict the pharmacology or tissue distribution of the human A3 adenosine receptor.

Although the human A1, A2a and A2b adenosine receptor cDNAs have been cloned, the tissue distribution of human adenosine receptor transcripts has not been previously presented. This patent
10 disclosure describes the characterization of a human A3 adenosine receptor subtype, the pharmacological profile of the human A3 adenosine receptor, and the tissue distribution of human A1, A2a, A2b and A3 adenosine receptor transcripts.

Based on the use of these cloned receptors, an assay has
15 been described to identify adenosine receptor agonists and antagonists and determine their binding affinity (see GB 2 264 948 A, published 9/15/93; see also R.F. Bruns, et al., (1983) Proc. Natl. Acad. Sci. USA 80:2077-2080; R.F. Bruns, et al., (1986) Mol. Pharmacol. 29:331-346; M.F. Jarvis, et al. (1989) J. Pharma. Exp. Therap. 251:888-893; K.A.
20 Jacobson et al., (1989) J. Med. Chem. 32:1043-1051).

Adenosine receptor agonists, antagonists and binding
enhancers have been identified and implicated for usage in the treatment of physiological complications resulting from cardiovascular,
pulmonary, renal and neurological disorders. Adenosine receptor
25 agonists have been identified for use as vasodilators ((1989) FASEB J. 3(4) Abs 4770 and 4773, (1991) J. Med. Chem. (1988) 34:2570), antihypertensive agents (D.G. Taylor et al., FASEB J. (1988) 2:1799), and anti-psychotic agents (T.G. Heffner et al., (1989) Psychopharmacology 98:31-38). Adenosine receptor agonists have been
30 identified for use in improving renal function (R.D. Murray and P.C. Churchill, (1985) J. Pharmacol. Exp. Therap. 232:189-193). Adenosine receptor allosteric or binding enhancers have shown utility in the treatment of ischemia, seizures or hypoxia of the brain (R.F. Bruns, et al. (1990) Mol. Pharmacol. 38:939-949; C.A. Janusz, et al., (1991)

- 5 -

Brain Research 567:181-187). The cardioprotective agent, 5-amino-4-imidazole carboxamide (AICA) ribose has utility in the treatment of ischemic heart conditions, including unstable angina and acute myocardial infarction (H.E. Gruber, et al. (1989) Circulation 80: 1400-1414).

8-phenylxanthines, methods of their synthesis and their use in human and veterinary therapy for conditions associated with the cell surface effects of adenosine have been described (EP 0 203 721, published 12/3/86). However, this publication is silent as to adenosine receptor subtypes and subtype specificity of disclosed compounds. In WO 90/00056, a group of 1,3-unsymmetrical straight chain alkyl-substituted 8-phenylxanthines were described as being potent bronchodilators. This disclosure is likewise silent as to the subtype specificity of disclosed compounds.

Methods of treating conditions related to the physiological action of adenosine have, to date, proven inferior due to the presence of multiple subtypes present in the animal tissue utilized (R.F. Bruns et al., (1986) Mol. Pharm. 29:331-346) and the differences between species in the affinity for adenosine analogs and the physiological effects of adenosine (Ukera, et al., (1986) FEBS Lett, 209:122-128).

Through the use of homoeonous, recombinant adenosine receptors, the identification and evaluation of compounds which have selectivity for a single receptor subtype has now been accomplished. Moreover, because of the variable effects of adenosine documented in other species, the utilization of human adenosine receptor subtypes is advantageous for the development of human therapeutic adenosine receptor agonists, antagonists or enhancers. The instant patent disclosure defines compounds which unexpectedly exhibit selective binding affinity for the human A3 adenosine receptor and therefore provides a method of using such compounds which overcomes the disadvantages of using compounds of uncharacterized specificity, by specifically blocking the activities mediated through the activation of the A3 receptor subtype without substantially blocking the activities of the other adenosine receptor subtypes.

- 6 -

BRIEF DESCRIPTION OF THE DRAWINGS

- 5 Figure 1 Full length amino acid sequence of human A1 adenosine receptor.
- Figure 2A-B Full length nucleotide sequence of the cloned human A1 adenosine receptor complementary DNA depicted from the 5' to 3' terminus.
- 10 Figure 3 Full length amino acid sequence of human A2a adenosine receptor.
- Figure 4A-B Full length nucleotide sequence of cloned human A2a adenosine receptor complementary DNA depicted from the 5' to 3' terminus.
- 15 Figure 5 Full length amino acid sequence of human A2b receptor.
- Figure 6A-B Full length nucleotide sequence of cloned human A2b adenosine receptor complementary DNA depicted from the 5' to 3' terminus.
- 20 Figure 7 Saturation binding of [³H]-cyclohexyladenosine (CHA) to human A1 adenosine receptor in COS7 assay.
- 25 Figure 8 Saturation binding of [³H]-CGS21680 to human A2a adenosine receptor in COS7 assay.
- Figure 9 Full length amino acid sequence of human A3 adenosine receptor.
- 30 Figure 10A-B Full length nucleotide sequence of the cloned human A3 adenosine receptor complementary DNA depicted from the 5' to 3' terminus.

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- 7 -

Figure 11 (A) Equilibrium binding of ^{125}I -ABA to membranes prepared from A3 stable transfected CHO cells shows specific (\bullet) and nonspecific (\circ) binding. Nonspecific binding was measured in the presence of $1\ \mu\text{M}$ I-ABA.

5 (B) Scatchard transformation of the specific binding.

Figure 12A-B Competition by agonists and antagonists for ^{125}I -ABA binding to membranes prepared from stably transfected CHO cells expressing the human A3 adenosine receptor. Agonists (top panel), (\bullet) NECA, (\circ) R-PIA, (\bullet) CPA, (\circ) S-PIA; antagonists (bottom panel), (\bullet) I-ABOPX, (\bullet) BW-A1433, (\circ) XAC, (\circ) DPCPX.

10

15

Figure 13A-F Competition by antagonists of NECA-inhibited cyclic AMP accumulation in CHO cells stably expressing the human A3 adenosine receptor. Dose response curves to NECA measured in the absence or presence of two concentrations of BW-A1433, XAC and I-ABOPX. The ED_{50} 's were used to construct Schild plots. Each figure is representative of 2-3 experiments.

20

Figure 14A-B Northern blot analysis of the four human adenosine receptor subtypes. (A) $5\ \mu\text{g}$ poly(A) $^{+}$ RNA from various human tissues probed with HS-21a. The two blots shown were transferred, hybridized and exposed separately. (B) $7.5\ \mu\text{g}$ poly(A) $^{+}$ RNA from various human tissues probed with either A1, A2a, or A2b. Each blot was transferred and exposed separately.

25

30

Figure 15(A)-(E) Structures of xanthine derivatives.

Figure 16 An image showing the abluminal surface of an arteriole

- 8 -

after staining with methylene blue, clearly labeling adherent mast cells, some of which are degranulated from prior exposure to adenosine.

- 5 Figure 17 Change in microvessel diameter with A3 agonist.
- Figure 18 Peak concentration-response curves for untreated vessels
 exposed to compound 48/80 (n = 4). Values are mean
 \pm s.e. for the peak luminal diameter attained within one
10 minute of compound 48/80 application.
- Figure 19 Peak concentration-response curves for control arterioles
 and arterioles pretreated with cromolyn. "Initial" indicates
 baseline tone before pretreatment. *, p< 0.05.
- 15 Figure 20 Abolition of agonist induced contractile response by pre-
 treatment with an adenosine A3 receptor specific
 antagonist.

20 SUMMARY OF THE INVENTION

 The invention concerns the use of compounds, identified through the use of recombinant human adenosine receptors A1, A2a, A2b and A3, and functional assays, to specifically modulate the physiologic role of adenosine activation of its various receptors.

- 25 The human A3 adenosine receptor was cloned from a striatal cDNA library using a probe derived from the homologous rat sequence. The cDNA encodes for a protein of 318 amino acids and exhibits 72% and 85% overall identity with the rat and sheep A3 adenosine receptor sequences, respectively. Specific and saturable
30 binding of the adenosine receptor agonist, ^{125}I -N6-aminobenzyladenosine (^{125}I -ABA) was measured on the human A3 receptor stably expressed in CHO cells with a $K_D = 10$ nM. The potency order for adenosine receptor agonists was determined to be N-ethylcarboxamidoadenosine (NECA) \geq R-phenylisopropyladenosine (R-

- 9 -

PLA) > N⁶-cyclopentyladenosine (CPA) > S-phenylisopropyladenosine (S-PIA). The human receptor was found to be blocked by xanthine antagonists. A partial listing of the pharmacology is that the potency order of antagonists is I-ABOPX > 1,3-dipropyl-8-(4-acrylate) phenylxanthine (BW-A1433) ≥ xanthine amino cogener (XAC) >> 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). Adenosine, NECA, R- and S-PIA and CPA inhibited forskolin-stimulated cAMP accumulation by 30-40% in the stably transfected CHO cells; I-ABA is a partial agonist. When measured in the presence of antagonists, the dose response curves of NECA-induced inhibition of forskolin-stimulated cAMP accumulation were right-shifted. Antagonist potencies determined by Schild analyses correlated well with those established by competition for radioligand binding. The tissue distribution of transcripts for all of the human adenosine receptor subtypes was compared. The A₃ adenosine receptor transcript is widespread, and in contrast to the A₁, A_{2a} and A_{2b} transcripts, the most abundant expression is found in the lung and liver. By comparison, the rat A₃ adenosine receptor transcript is primarily expressed in testis and the sheep transcript is most abundant in the lung, spleen and pineal. The human tissue distribution of A₃ mRNA is more similar to the widespread profile found in sheep than to the restricted profile found in the rat. Numerous physiological effects of adenosine may be mediated by A₃ adenosine receptors in man.

25

ABBREVIATIONS

[³H]-CHA, [³H]-cyclohexyladenosine; [³H]-NECA, [³H]-5'-N-ethylcarboxamido-adenosine; ¹²⁵I-ABA, N⁶-(4-amino-3-¹²⁵iodobenzyl)adenosine; ¹²⁵I-APNEA, N⁶-2-(4-amino-3-¹²⁵iodophenyl)ethyladenosine; NECA, N-ethylcarboxamidoadenosine; CGS21680, 2-[4-(2-carboxyethyl)phenyl]ethylamino-5'-N-ethylcarboxamidoadenosine; (R,S)-PIA, (R,S)-N⁶-phenyl-2-propyladenosine; CPA, N⁶-cyclopentyladenosine; I-ABOPX, (3-(3-iodo-4-aminobenzyl)-8-(4-oxacetate)phenyl-1-propylxanthine; BW-A1433, 1,3-dipropyl-8-(4-acrylate)phenylxanthine; XAC, xanthine

- 10 -

amine cogener; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; GTP γ S, guanosine 5'-O-3-thiotriphosphate; Gpp(NH)p, 5'-guanylimidodiphosphate; G protein, guanine nucleotide-binding proteins.

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10 DETAILED DESCRIPTION OF THE INVENTION

This invention provides a method for achieving specific blockade of the A3 subtype of the adenosine receptor. We have discovered that adenosine, adenosine metabolites and other A3 adenosine receptor agonists induce mast cell degranulation in an animal model and
15 that this can be prevented by selective antagonists of the A3 receptor. The release of enzymes, bioactive amines and arachidonic acid metabolites following mast cell activation causes vasoconstriction, edema, leukocyte accumulation, and ultimately, tissue damage. Mast cell degranulation is a component of: myocardial reperfusion injury,
20 hypersensitivity reactions (asthma, allergic rhinitis, and urticaria), ischemic bowel disease, autoimmune inflammation, and atopic dermatitis. The invention consists of the use of any of a series of highly specific A3 adenosine receptor antagonists to treat or prevent these diseases and pathologic effects that result from mast cell degranulation.

25 Other physiologic effects induced through activation of the A3 adenosine receptor are also amenable to modulation through blockade of A3 adenosine mediated responses in basophiles, eosinophiles and other immune cells. Mast cell degranulation is clearly involved in the pathophysiology of allergies such as asthma. Autoimmune diseases
30 are also characterized by immune reactions which attack targets, including self-proteins in the body such as collagen, mistaking them for invading antigens. The resulting damage, caused at least in part by mast cell degranulation, is amenable to treatment by the method of this invention which comprises administration of selective A3 adenosine

- 13 -

receptor antagonists effective to inhibit mast cell degranulation. Among these types of diseases, all of the following, but not limited to these, are amenable to treatment by the method of this invention:

5 Addison's disease (adrenal), autoimmune hemolytic anemia (red cells),
Crohn's disease (gut), Goodpasture's syndrome (kidney and lungs),
Grave's disease (thyroid), Hashimoto's thyroiditis (thyroid), idiopathic
thrombocytopenic purpura (platelets), Insulin-dependent diabetes militus
(pancreatic beta cells), multiple sclerosis (brain and spinal cord),
10 myasthenia gravis (nerve/muscle synapses), Pemphigus vulgaris (skin),
pernicious anemia (gastric parietal cells), poststreptococcal
glomerulonephritis (kidney), psoriasis (skin), rheumatoid arthritis
(connective tissue), scleroderma (heart, lung, gut, kidney), Sjogren's
syndrome (liver, kidney, brain, thyroid, salivary gland), spontaneous
infertility (sperm), systemic lupus erythematosus (DNA, platelets, other
15 tissues).

The method of this invention provides a means for preventing or treating disease states associated with vascular constriction induced through activation of the A3 subtype of the adenosine receptor. The method comprises contacting said receptor in the vasculature with
20 an amount of a compound which selectively blockades activation of the A3 adenosine receptor subtype. In one embodiment of the invention, this blockade occurs on granulocytes, including mast cells, exhibiting the A3 adenosine receptor. In one embodiment of the invention, xanthine or a xanthine derivative is used to effect a reduction in
25 vasoconstriction in the vasculature without any substantial effect (binding or blockade) of the A1, A2a or A2b subtypes of the adenosine receptor. The method extends to the treatment or prevention of disease states mediated through activation of the A3 subtype of the adenosine receptor on mast cells. Prevention of mast cell degranulation through
30 blockade of the A3 subtype of the adenosine receptor by contacting mast cells with an inhibitory effective amount of a xanthine or xanthine derivative specific for the A3 receptor subtype therefore also forms part of this invention. Disease states associated with A3 adenosine receptor activation and mast cell degranulation include, but are not

- 14 -

limited to asthma, myocardial reperfusion injury, allergic reactions including but not limited to rhinitis, asthma, poison ivy induced responses, urticaria, scleroderma, arthritis, and inflammatory bowel diseases.

5 Compounds having specific affinity for the A3 adenosine receptor subtype are identified through the pharmacology displayed in binding to isolated receptors of the various subtypes. Many compounds have been identified broadly as antagonists of the adenosine receptor. However, the subtype specificity of these compounds, in particular the
10 pharmacology of the primate A3 receptor subtype, was not known prior to the instant patent disclosure. This disclosure defines xanthines and xanthine derivatives displaying potent and specific A3 subtype specificity. This disclosure also defines the unexpectedly similar tissue distribution and pharmacology of the human and sheep A3 receptors.
15 Because of this discovered similarity, it is now possible to utilize known sheep A3 pharmacology to predict primate pharmacology. In addition, this disclosure demonstrates the functional effects of using A3 specific compounds in blocking adenosine induced effects. Microvasculature
20 contraction is inhibited, intracellular cAMP reduction, which is normally induced by A3 receptor agonism, is blocked and mast cell degranulation is inhibited.

 A cDNA from human striatum designated HS-21a that encodes a human A3 adenosine receptor has been cloned. The cDNA is
25 homologous with rat (5,6) and sheep clones (7), and all three sequences encode receptors that couple adenosine induced inhibition of cAMP accumulation when stably expressed in CHO cells. ¹²⁵I-ABA, previously used as a radioligand for A1 adenosine receptors (12), was found to be suitable for detecting recombinant CHO cells expressing human A3
adenosine receptors.

30 The sheep A3 adenosine receptor transcript is widely distributed, with high levels found in lung and spleen and moderate levels found in brain, pineal and testis. In marked contrast, the rat A3 adenosine receptor transcript is found primarily in testis. Therefore, it was of great interest to determine the tissue distribution of the human

- 15 -

A3 adenosine receptor transcript. We have discovered that the human transcript is found to be more similar to the sheep than the rat homolog, with high expression in lung, moderate expression in brain and low expression in testis.

5 The rat A3 adenosine receptor differs from the human and sheep receptors in that it was reported not to bind the xanthine antagonists, XAC and DPCPX (6). The sheep and now the human A3 adenosine receptors have been found to bind both antagonists and also have high affinity for 8-substituted xanthines having acidic substitutions.
10 At the same time that the acidic substitutions increase the binding affinity of these compounds for the A3 receptor, they decrease the affinity for the A1, A2a and A2b subtypes. A limited number of xanthine analogs were evaluated in the pharmacological characterization of the rat A3 receptor and it was reported that these compounds do not
15 significantly bind to the rat A3 receptor. The opposite is true in the case with the sheep A3 homolog. We have now discovered that the human A3 receptor has a high affinity for this class of compounds.

 A few significant differences in ligand binding and transcript expression exist between the human and sheep receptors. I-
20 ABA appears to be a full and partial agonist, respectively, for lowering cyclic AMP in CHO cells transfected with sheep and human receptors. The human receptor has higher affinity for CPA in comparison to the sheep receptor. An agonist affinity order of I-ABA > NECA > R-PIA > S-PIA >> CPA was established for the sheep receptor (7). By
25 comparison, the human receptor has a generally higher affinity for all of the agonists and a preference for CPA over S-PIA, resulting in an agonist profile of I-ABA > NECA > R-PIA > CPA > S-PIA.

 The antagonist affinity order profiles are similar between human and sheep receptors, however, the human homolog exhibits a
30 higher affinity for XAC. Since 8-phenylxanthines with para-acidic residues were found to bind with high affinity to sheep A3 adenosine receptors (7), we evaluated acidic 8-phenylxanthines as human A3 adenosine receptor antagonists. Included among these are compounds with various 3-substitutions that were evaluated previously as potent

- 16 -

antagonists of A1 adenosine receptors (13). One of these compounds, I-ABOPX, was found to have the highest affinity as an A3 adenosine receptor antagonist, with a K_i of 18 nM for the human receptor and 2 nM for the sheep receptor.

5 The potency order profiles of agonist and antagonist binding to the A3 receptor differ substantially from the profiles established for the other cloned human adenosine receptor subtypes (4). Pharmacologically, the A3 receptors appear to more closely resemble A1 than the A2 adenosine receptors. This is consistent with the fact that
10 the human A3 sequence is more similar to the A1 subtype (identity score 49%) than to the A2a and A2b subtypes. All subtypes of human adenosine receptors are blocked by xanthine antagonists such as BW-A1433, XAC and DPCPX, but differ in their affinities and potency order profiles for these ligands. The A1 subtype has high affinity for
15 agonists with saturated rings in the N⁶ position of the adenine ring, and xanthine antagonists with saturated rings in the C⁸ position.

 The human A3 receptor has a slightly higher affinity for ligands with unsaturated than saturated rings in the N⁶ position of agonists (R-PIA > CPA) and in the C⁸ position of xanthines (BW-
20 A1433 and XAC > DPCPX). On the basis of structure-activity relationships of A1 agonists and antagonists, a model has been proposed in which the N⁶-substituents of the adenine ring can be superimposed upon the C⁸-regions of xanthines (27,28). A similar relationship has
25 been suggested to exist for the sheep A3 receptor since parallel changes in potency for agonists and antagonists were observed when the N⁶ and C⁸ positions, respectively were substituted with unsaturated or saturated rings (7). The human A3 receptor was found to also exhibit
corresponding changes in agonist and antagonist affinities when saturated substitutions were introduced at the N⁶ and C⁸ positions.

30 In a preferred embodiment of this invention, an A3 adenosine receptor antagonist will have a pK_i for the A3 subtype of 7 or greater, and a pK_i for other adenosine receptor subtype of 6 or less. From this disclosure, it will be apparent to those skilled in the art that xanthine compounds having the following characteristics are preferred:

- 17 -

An acidic, aromatic substitution at the 8 position of the xanthine (the acidity decreases affinity to A1 subtype to below a pKi of about 6, and the aromatic substitution reduces the affinity for the A2 subtype to below about 6). By further modifying the xanthine to include an aromatic, and preferably a halogenated aromatic at the xanthine 3 position, these trends in binding affinity are accentuated.

A listing of the antagonist profiles for a number of these compounds is shown in Table 1, and the structures of these compounds is shown in the figures:

TABLE 1

Human A3		HUMAN A1
Drug	Pki	Pki
AGONISTS		
1. LABA	8.0	8.80
2. NECA	7.59	•
3. R-PIA	7.47	8.53
4. APNEA	7.22	8.05
5. CPA	6.80	9.15
6. S-ENBA	4.86	•
ANTAGONISTS		
1. LABOPX	8.13	6.12
2. XAC	7.31	•
3. BW-A1433	7.00	•
4. SPX	5.99	•
5. CPX	5.36	9.15
6. SPT	4.95	5.60
7. THEO.	4.23	•

- 18 -

This human data can be compared with sheep data as follows in table 2:

5

TABLE 2

Sheep S-17 "A3" (CHO cells)
Kd (125 I ABA) = 5.5 nM
Bmax = 527 fmol/mg protein

10

Drug	pKi
------	-----

1. BW-A934	9.04
------------	------

2. LABOPX	8.23
-----------	------

3. BW-A857	7.71
------------	------

15

4. BW-1433	7.69
------------	------

5. ABOPX	7.65
----------	------

6. BW-B40	7.48
-----------	------

7. BW-215	7.26
-----------	------

8. XAC	6.77
--------	------

9. CPX or	4.30
-----------	------

20

DPCPX	
-------	--

Table 3. Comparison of agonist and antagonist affinities for the human A3 adenosine receptor determined in binding and functional cAMP assays.

25

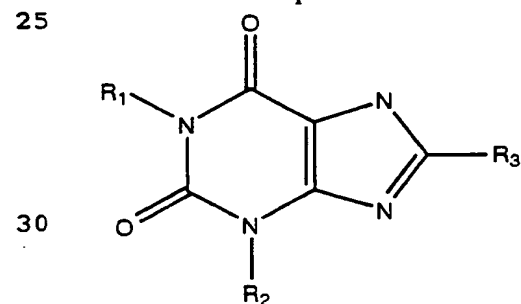
30

- 19 -

		BINDING	cAMP
		<u>pK_D or pK_i</u>	<u>pK_A or pK_i</u>
Agonists			
5	LABA	8.0	7.46
	NECA	7.59	6.89
	R-PIA	7.47	6.14
	CPA	7.05	6.8
	S-PIA	6.49	6.04
10	Antagonists		
	LABOPX	7.74	8.13
	XAC	7.15	7.66
15	BW-A1433	7.26	7.0
	DPCPX	6.12	5.36

20 The log ED₅₀ (pK_A) of agonists to inhibit cAMP accumulation and the K_i of antagonists and I-ABA was determined by Schild analysis.

25 From these studies, the use of a class of xanthines and their derivatives having the following characteristics is defined as having selective binding properties for the primate (including human) A₃ adenosine receptor:



- 20 -

wherein R₁, R₂, and R₃, independently, are as defined below:

	<u>R₁</u>	<u>R₂</u>	<u>R₃</u>
5	alkyl alkenyl cycloalkyl	aryl substituted aryl hetero-aryl substituted hetro-aryl	acidic aryl substituted acidic aryl

10 wherein:

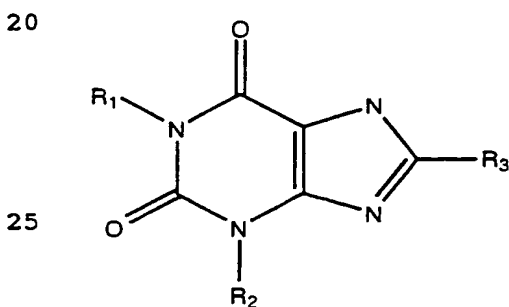
alkyl, alkenyl, cycloalkyl is substituted or unsubstituted

aryl is benzyl, phenyl;

substituted aryl is an aryl substituted with an alkyl, amino or halogen;
and

15 acidic aryl is an aryl substituted with a carboxylate, oxyacetate, acrylate, sulphonate, phosphonate, or tetrazol.

In a preferred embodiment of this invention, the xanthine
is:

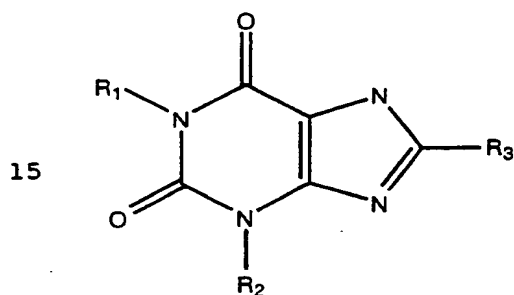


- 21 -

wherein R₁, R₂, and R₃, independently, are as defined below:

	<u>R₁</u>	<u>R₂</u>	<u>R₃</u>
5	lower alkyl	benzyl halogenated benzyl amino-benzyl halogenated amino-benzyl.	benzyl-acid

10 In a further embodiment, the xanthine is:



20 wherein R₁, R₂, and R₃, independently, are as defined below:

	<u>R₁</u>	<u>R₂</u>	<u>R₃</u>
	-C ₃ H ₇	-C ₃ H ₇	-CH ₂ -C ₆ H ₄ -O-acid
	-CH ₃	-benzyl	-CH ₂ -COO ⁻
25	-C ₂ H ₅	-halogenated benzyl -aminobenzyl -halogenated aminobenzyl	-indole

30

wherein said acid is -indole, -carboxylate, sulphonate, phosphonate. In specific embodiments of the invention, the xanthine is selected from the group consisting of IABOPX, BW-A1433, BW-934, BW-A215.

A comparison of the distribution among tissues of human adenosine receptor transcripts suggests that the A₁, A_{2a}, A_{2b} and A₃

- 22 -

subtypes are all expressed in a number of tissues, but the pattern of transcript distribution is variable. Within the four tissues analyzed, A1 and A2a adenosine receptor transcripts are highly expressed in the brain. In contrast, the human A3 adenosine receptor transcript is most
5 abundant in the lung and liver.

Prior to the instant patent disclosure, the physiological role of the A3 adenosine receptor was not defined. Recently, a receptor exhibiting an agonist pharmacological profile thought to be characteristic of the rat A3 subtype and insensitive to blockade by 8-
10 (para-sulphophenyl)theophylline, was suggested to mediate in vivo hypotension in the angiotensin II-supported circulation of the pithed rat (30). The effect of 8-(para-sulphophenyl)theophylline on the cloned rat A3 receptor has not been evaluated and further pharmacological characterization is required to determine if the rat receptor binds this
15 antagonist. 8-Para-sulphophenyltheophylline has broad action on A1, A2a and A2b adenosine receptor subtypes (2), and also blocks both the human and sheep A3 receptors. Another possible physiological role for the A3 adenosine receptor subtype in reproduction and spermatogenesis has been proposed on the basis of the abundant transcript found in rat
20 testis and the in situ localization of mRNA within the central luminal regions of seminiferous tubules where sperm maturation occurs (5). It is possible that A3 adenosine receptors also are involved in the maturation of sperm in human and ovine testis, since low to moderate levels of receptor transcript are found in these species, respectively.

25 The abundant mRNA observed in the human and sheep lung is evidence that the A3 adenosine receptor subtype mediates a physiological action in the pulmonary system. Adenosine has been shown to mediate both vasodilation and vasoconstriction (31,32) in the pulmonary vasculature. In asthmatics, but not in normal patients,
30 adenosine produces bronchoconstriction which can be antagonized by theophylline (33). The establishment of the pharmacological profile for the A3 receptor in both the human and the sheep, and the availability of subtype selective ligands facilitates the identification of the physiological functions mediated by the A3 adenosine receptor subtype and the

- 23 -

treatment of disease states mediated through agonism of this receptor subtype.

Adenosine has been shown to produce bronchoconstriction in asthmatics but not in nonasthmatics, demonstrating that adenosine plays a role in the etiology of this disease state (Cushly et al., 1984, AM. Rev. Respir. Dis. 129:380-384). Adenosine mediated bronchoconstriction in asthmatics is blocked by a combination of histamine and leukotriene antagonists (Bjorck et al., Am. Rev. Resp. Dis. 1992, 145:1087-1091). This indicates that adenosine acts by releasing histamine, leukotriene and other agents from mast cells or other cells that contain these allergic mediators. It is therefore within the ambit of this invention that compounds identified herein as being useful to selectively antagonize the A3 adenosine receptor are used in conjunction with other therapies. This includes co-administration of anti-histamine, leukotriene blockade or other anti-allergic mediator therapies and A3 specific antagonists. The presence of the rat A3 adenosine receptor on rat RBL-2H3 mast cells, activation of which results in potentiation of the histamine secretory response to antigen (Ramkuman et al., J. Biol. Chem. 268:16887-16890, 1993) is evidence in the rat of the role of these receptors in that animal. However, because of the reportedly different pharmacology of the rat A3 receptor as opposed to that of the human and sheep A3 receptors, it could not be predicted that the A3 receptor was significant in primates including humans.

The instant patent disclosure provides evidence that the blockade of A3 adenosine receptor mediated action in the vasculature is useful to treat and prevent disease states in humans. reports that adenosine potentiates the release of granule contents from mast cells isolated from rat peritoneum (Lohse et al., N.-S. Arch. Pharmacol. 335:555-560, 1987; Marquardt et al., J. Immunol. 120:871-878, 1978), and that mast cell degranulation causes constriction in some vascular beds resulting in C5a-induced myocardial ischemia (Ito et al., Am. J. Physiol. 264 (Heart Circ. Physiol. 33):H1346-H1354, 1993), mast cell-dependent inflammation (Raud, J., Acta. Physiol. Scand. 135 (Suppl.

- 24 -

578):1-58, 1989), brain arteriole diameter constriction (Rosenblum, W. I., Brain Res. 49:75-82, 1973), and the release of allergic mediators (Ramkumar, et al., J. Biol. Chem. 268:16887-16890, 1993).

Accordingly, if a method were invented to specifically inhibit mast cell degranulation, a treatment or preventative method would be demonstrated for all of the above known and many yet to be defined disease states associated with adenosine induced mast cell degranulation.

The trigger for mast cell degranulation is usually thought to be an allergen. Allergens are endocytosed by macrophages and degraded. The resulting fragments are displayed on T lymphocytes. B lymphocytes are stimulated to mature into plasma cells which are able to secrete allergen-specific molecules known as immunoglobulin E, (IgE). These antibodies attach to receptors on mast cells in tissue and on basophils circulating in blood - to trigger degranulation (see L. Lichtenstein, Sci. Am. 269:116-125, 1993). As described below, our data shows that activation of A3 adenosine receptors can produce mast cell degranulation and enhance the effect of allergens. Adenosine and antigens trigger an influx of calcium to induce mast cell granules to release their contents and promote synthesis and release of cytokines, prostaglandins and leukotrienes. The various chemicals released by mast cells are responsible for many allergic symptoms. Long term release of these chemicals can induce basophils, eosinophils, and other cells flowing through blood vessels to migrate into the tissue. Migration is promoted due to the expression and activation of adhesion molecules on the circulating cells and on vascular endothelial cells. The circulating cells adhere to the endothelial cells, roll among them, and eventually cross into the surrounding matrix. These recruited cells secrete chemicals of their own that damage tissue. Thus, there are long term secondary effects which may also be prevented by specific blockade of mast cell degranulation.

We have shown that specific blockade of the A3 subtype of the adenosine receptor is effective to block the vasoconstrictive response induced through adenosine activation of this receptor subtype. We have also shown that use of a specific inhibitor of the A3 adenosine receptor

- 25 -

subtype is effective to inhibit effects induced by adenosine mediated mast cell degranulation, and have therefore shown that disease states including but not limited to myocardial ischemia (Ito et al., Am. J. Physiol. 264 (Heart Circ. Physiol. 33):H1346-H1354, 1993), mast cell-
5 dependent inflammation (Raud, J., Acta. Physiol. Scand. 135 (Suppl. 578):1-58, 1989), brain arteriole diameter constriction (Rosenblum, W. I., Brain Res. 49:75-82, 1973), and the release of allergic mediators (Ramkumar, et al., J. Biol. Chem. 268:16887-16890, 1993), are all
10 amenable to prevention and treatment by contacting A3 receptor bearing mast cells with an amount of a selective A3 inhibitor effective to prevent mast cell degranulation.

In another embodiment of this invention, the method is directed to prevention or treatment of myocardial ischemia/reperfusion. The basis of this application of the method is that a period of
15 myocardial ischemia followed by reperfusion produces damage to the myocardium. Part of this damage may be secondary to mast cell degranulation triggered by adenosine during ischemia. This suggests that A3 adenosine receptor antagonists may be useful for the treatment of patients prone to reperfusion injury. This includes patients with
20 coronary artery diseases in general, and patients about to have occluded arteries opened (reperfused) by various interventions (coronary artery bypass grafts, angioplasty or thrombolytic therapy). Adenosine-induced mast cell degranulation during a period of transient ischemia may be responsible for the phenomenon of preconditioning (i.e. a transient
25 ischemic episode reduces myocardial damage resulting from a subsequent prolonged ischemic episode). Accordingly, mast cells are temporarily depleted of damaging mediators during the preconditioning period. These observations are supported by reports suggesting that mast cell degranulation is involved in ischemia/reperfusion injury (see
30 for example Heller, L.J. and Regal, J.F., Effect of adenosine on histamine release and atrioventricular conduction during guinea pig cardiac anaphylaxis. Circ. Res. 62:1147-1158, 1988. Conclusion: increases in levels of endogenous adenosine during cardiac anaphylaxis contributed to the development of atrioventricular conduction delays

- 26 -

and that increases in levels of adenosine before antigen challenge may increase the amount of histamine released during cardiac anaphylactic reactions; Wolff, A.A. and Levi, R., Ventricular arrhythmias parallel cardiac histamine efflux after coronary artery occlusion in the dog. Agents and Actions 25:296-306, 1988. Conclusion: During acute myocardial ischemia, the coronary sinus histamine concentration increases simultaneously with the development of early ischemic ventricular arrhythmias and in proportion of their severity; Keller, A.M. Clancy, R.M., Barr, M.L. Marboe, C.C. and Cannon, P.J., Acute reoxygenation injury in the isolated rat heart: role of resident cardiac mast cells. Circ. Res. 63:1044-1052, 1988. conclusion: The isolated crystalloid-perfused rat heart is not a leukocyte-free preparation and mast cells resident to the heart play an important role in acute reoxygenation injury; Jolly, S.R., Abrams, G.D., Romson, J.L., Bailie, M.B. and Lucchesi, B.R., Effects of lodoxamide on ischemic reperfused myocardium. J. Cardiovas. Pharmacol. 4:441-448, 1982. conclusion: Lodoxamide, a drug that acts to inhibit mast cells degranulation, reduces myocardial ischemic injury; Ito, B.R., Engler, R.L., Del Balzo, U., Role of cardiac mast cells in complement C5a-induced myocardial ischemia. Am. J. Physiol. 33:H1346-H1354, 1992. conclusion: Cardiac mast cells are involved in complement-induced release of vasoactive eicosanoids, including TxA₂).

The human A₁, A_{2a}, A_{2b} and A₃ receptor subtype cDNAs were subcloned into the expression vectors pSVL (PHARMACIA), CMV5 (Mumby, et al. 1990, PNAS, 87:728-732) or pREP (INVITROGEN). Transient expression in COS7 cells (monkey kidney cell line, ATCC CRL 1651, ATCC, Rockville, MD) was accomplished by transfection of the cloned adenosine receptor cDNAs under the control of the SV40 promoter into mammalian cells (e.g., COS7). Membranes prepared from the transfected cells were utilized for the determination of binding affinity, selectivity and specificity of the human adenosine receptors for various ligands. Stable expression of the human adenosine receptors in mammalian cells (e.g., CHO, HEK 293) was achieved after integration of the transfected cDNA into the

- 27 -

chromosomes of the host cells. These stable cell lines constitutively express the cloned human adenosine receptors and can be propagated infinitely. Stable cell lines expressing the human adenosine subtype cDNAs individually can be used in the binding assay to measure the affinity and selectivity of the receptors for adenosine agonists, antagonists and enhancers.

Membranes prepared from transfected COS7 cells were utilized in a binding assay to measure the affinity of the human adenosine receptors for the radiolabeled adenosine agonists, [^3H]-cyclohexyladenosine (CHA), [^3H]-CGS21680 (2-(p-(2-carboxyethyl)-phenylamino)-5'-N-ethyl-carboxamidoadenosine), [^3H]-5'-N-ethylcarboxamido adenosine ([^3H]-NECA), or [^{125}I]-N⁶-aminobenzyl adenosine (^{125}I -ABA). Monolayer cell culture of transfected COS7 cells were dissociated with 1 mM EDTA in phosphate buffered saline and resuspended in 5 mM Tris, pH7.6/10 mM MgCl_2 . The cells were subjected to freeze-thaw lysis and the suspension was homogenized in a glass dounce homogenizer. The membranes were pelleted, resuspended in binding buffer, 50 mM Tris pH 7.6/10 mM MgCl_2 and incubated with adenosine deaminase before the binding assay. The binding assay was performed by incubating 50-100 μg of membranes with increasing concentrations of radiolabeled adenosine agonists. Bound ligand was separated from free ligand by filtration on a SKATRON CELL HARVESTER equipped with a receptor binding filtermat. Bound radioactivity was measured by scintillation counting. Substances which bind to or enhance binding to expressed human adenosine receptors in COS and CHO cells can be identified in competition binding assays with radiolabeled adenosine or xanthine analogs. For the competition binding assay, membranes were incubated with 5nM [^3H]-CHA 5nM [^3H]-CGS21680 or 10nM [^3H]-NECA and various concentrations of adenosine agonists or antagonists.

A transient expression system in *Xenopus* oocytes was established by microinjection of in vitro transcribed mRNA from the cloned adenosine receptor cDNAs. The expression system allows the measurement of the biological effects (i.e., changes in cAMP levels)

- 28 -

upon activation of the expressed adenosine receptors with ligand binding. The cAMP levels are measured by a radioimmunoassay (RIANEN kit, DuPont/NEN) using the acetylation protocol. Activation of the expressed receptors by ligand binding are coupled to either
5 increases or decreases in the intracellular cAMP levels dependent upon the subtype of adenosine receptor (Van Calker et al., (1979) J. Neurochem. 33:999-1003; Londos et al. (1980) Proc. Natl. Acad. Sci. USA 77:2551-2554). The activity of any potential adenosine receptor
10 agonist can be evaluated by measuring the changes in cAMP levels in oocytes injected with adenosine receptor mRNA but not in uninjected or negative control injected oocytes. The activity of any potential adenosine receptor antagonist can be evaluated by determining the inhibition of the cAMP response induced by adenosine in oocytes
15 injected with adenosine receptor transcripts but not negative control or uninjected oocytes. The changes in cAMP accumulation can alternatively be measured in stably transfected CHO cells expressing the human adenosine receptor subtypes.

The cAMP accumulation assay has a number of advantages over the binding assay established in the mammalian cell expression
20 system as a screen for adenosine receptor modulating agents. The assay allows the measurement of a biological effect (i.e., changes in cAMP levels) resulting from the activation of the expressed receptors by ligand binding. The native agonist adenosine is utilized in the assay to activate the expressed receptors. The functionality of additional adenosine
25 receptor subtypes identified by molecular cloning which may not have defined ligands for binding analysis can be evaluated with the natural agonist and without prior identification of a selective, high affinity, radiolabeled ligand.

According to the method of this invention, an adenosine A3
30 specific antagonist is administered in an amount effective to induce blockade of the receptor. The higher the affinity of the antagonist for the receptor, the lower the required dosage. Compounds having a pKi of greater than about 7 for the A3 receptor and below about 6 for other adenosine receptor subtypes, may be administered by any effective

- 29 -

means to achieve either localized or systemic contact of the antagonist with target A3 adenosine receptors. This might include intraveous, intramuscular, intrasynovial, intranasal, nebulized intrapulmonary, intraperitoneal or other common means for administration of therapeutic compounds. Dosages of between about 1 µg/kg and 10 mg/kg are envisioned, as necessary, to achieve the desired effect of A3 adenosine receptor blockade.

The following examples are provided to further define but not to limit the invention defined by the foregoing description and the claims which follow:

EXAMPLE 1

STEP A:

In the first step of obtaining the partial cDNAs encoding the human A1 and A2a adenosine receptors, total RNA was extracted by homogenizing 2.3g human ventricle in 20 ml 5M guanidine isothiocyanate, 0.1M sodium citrate, pH 6.3, 1mM EDTA, pH 7.0, 5% beta-mercaptoethanol, and 0.5% sodium lauryl sarcosinate. The homogenate was centrifuged for 10 min. at 10,000 rpm and the resulting supernatant was layered onto a cushion of 5.7M CsCl/0.1M EDTA, pH 7.0. After 20 hrs. of centrifugation at 24,000 rpm, the resulting pellet was precipitated one time and then passed over an oligo(dT)-cellulose (PHARMACIA, Piscataway, NJ) column to isolate poly(A)⁺ RNA.

An oligo(dT) primed library was synthesized from 5 µg of the poly(A)⁺ human ventricle RNA using the YOU-PRIME cDNA SYNTHESIS KIT (PHARMACIA, Piscataway, NJ). See Gubler and Hoffman *Gene* 25:263 (1983). The resulting double-stranded cDNA was ligated into λgt10 EcoRI arms (PROMEGA, Madison, WI) and packaged according to the GIGAPACK II GOLD PACKAGING EXTRACT protocol (STRATAGENE, La Jolla, CA). See Huynh et al. (1985) DNA Cloning Techniques: A Practical Approach, IRL Press, Oxford, p.49 and Kretz et al. *Res.* 17:5409.

- 30 -

The *E. coli* strain C600Hfl (PROMEGA, Madison, WI) was infected with library phage, plated on agar plates, and incubated at 37°C. The phage DNA was transferred to HYBOND-N nylon membranes (AMERSHAM, Arlington Heights, IL) according to the
5 manufacturer's specifications.

Synthetic probes were constructed from overlapping oligonucleotides (A1 probe: 62+63, A2 probe: 52+53; see Table I for their sequences) based on the published dog A1 (RDC7) and A2a(RDC8) sequences (F Libert, et al,(1989) Science 244:569-572).
10 The oligonucleotides were annealed and filled-in with $\alpha^{32}\text{P}$ -dCTP (NEN, Wilmington, DE) and Klenow enzyme. The filters were hybridized with the appropriate probe in 5XSSC, 30% formamide, 5XDenhardt's solution, 0.1% SDS, and 0.1mg/ml sonicated salmon sperm DNA at 42°C, overnight. Following hybridization the filters
15 were washed to a final stringency of 6XSSC at 50°C and exposed to X-OMAT AR film (KODAK, Rochester, NY) at -70°C. The resulting positives were plaque purified by two additional rounds of plating and hybridization. Insert DNA was excised with NotI and ligated into NotI digested pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA).
20 (Genebank # 52327) DNA sequences were determined by the SEQUENASE protocol (USBC, Cleveland, OH). See Tabor and Richardsaon, J. Biol. Chem. 264 pp 6447-6458. Two clones were isolated in these screens. The human ventricle A1 cDNA (hva1-3a) and human ventricle A2a cDNA (hva2-13) contain portions of coding
25 sequences for proteins homologous to the reported dog A1 and A2a cDNAs, respectively. The coding region of the human A1 clone corresponds to nucleotides 482 through 981 (Figures 2A and 2B) and is 92% identical to the dog A1 sequence at the nucleotide level. The coding region of the human A2a clone corresponds to nucleotides 497
30 through 1239 (Figure 4A and 4B), and is 90% identical to the dog A2a sequence at the nucleotide level.

- 31 -

STEP B:

The human ventricle A1 adenosine receptor partial cDNA (hvA1-3a) is a 543 bp NotI fragment containing 23 bp 3' untranslated sequence and is 460 bp short of the initiation methionine based on sequence homology to the dog A1 cDNA. A modification of the 5' RACE (rapid amplification of cDNA ends) method (MA Frohman et al, (1988), Proc. Natl. Acad. Sci. USA, 85:8998-9002) was used to generate the 5' coding region of the cDNA. First strand cDNA was synthesized from 1 µg of the human ventricle poly(A)⁺ RNA in a total volume of 40 µl containing 50mM Tris, pH 8.0, 140mM KCl, 10mM MgCl₂, 10mM DTT, 15mM each dNTP, 20 units RNasin (PROMEGA, Madison, WI), 20pmol human primer 79 (see Table I), and 9.2 units AMV reverse transcriptase at 37°C for 2 hrs. The reaction was then diluted to 120 µl with 0.5 mM Tris, pH 7.6/0.05 mM EDTA and passed through a SEPHACRYL S-300 SPUN COLUMN (PHARMACIA, Piscataway, NJ). The product in the column effluent was polyadenylated in 100mM potassium cacodylate, pH 7.2, 2mM CoCl₂, 0.2mM DTT, 0.15mM dATP, and 14 units terminal deoxynucleotidyl transferase in a total volume of 31 µl for 10 min. at 37°C. The reaction was terminated by heating at 65°C for 15 min. and then diluted to 500 µl with 10 mM Tris, pH 8.0/1 mM EDTA (TE).

Ten µl of the poly(A)-tailed first strand cDNA was used as template in a primary PCR amplification reaction according to the GENEAMP protocol (PERKIN ELMER CETUS, Norwalk, CT; see Saiki et al. (1988) Science 239:487-491) containing 10pmol primer 70, 25pmol primer 71, and 25pmol human primer 80 (see table I) in a total volume of 50 µl. Primer 70 is 5'-gactcgagtcgacatcga(t)₁₇, primer 71 is 5'-gactcgagtcgacatcga, and both are based on MA Frohman, et al (1988), Proc. Natl. Acad. Sci. USA, 85:8998-9002. One cycle of PCR was performed of 1 min at 95°C, 2 min at 50°C, 40 min at 72°C, followed by 40 cycles of 40 sec at 94°C, 2 min at 56°C, 3 min at 72°C. The primary PCR amplification reaction product was electrophoresed through a 1.4% agarose gel and an area corresponding to approximately 600 bp was excised. The gel slice was melted and 1 µl was used as

- 32 -

template in a secondary PCR amplification reaction containing 100pmol primer 71 and human primer 81 (see Table I) for 30 cycles of 1 min at 94°C, 2 min at 56°C, 3 min at 72°C. The secondary PCR amplification product was digested with EcoRI and Sall and electrophoresed on a 1.4% agarose gel. An area corresponding to 500-600bp was excised and ligated into EcoRI/Sall digested pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA). The sequence of the 515 bp PCR product (5'HVA1-9) was determined by the SEQUENASE protocol (USBC, Cleveland, OH). The partial human ventricle A1 cDNA and the PCR product contain overlapping sequence and represent the complete coding region for the human A1 receptor, including 14 and 23 bp of 5' and 3' untranslated sequences, respectively. The sequence of the human A1 adenosine receptor cDNA so identified, is shown in Figures 2A and 2B.

STEP C:

A probe was generated by Klenow enzyme extension, including $\alpha^{32}\text{P}$ -dCTP, of annealed oligonucleotides 62 and 63, and used to screen a human kidney cDNA library (CLONTECH, Palo Alto, CA). E. coli strain C600hfl (PROMEGA, Madison, WI) was infected with library phage and grown overnight on agar plates at 37°C. Phage DNA was transferred to HYBOND-N nylon filters according to the manufacturer's protocol (AMERSHAM, Arlington Heights, IL). The probe was incubated with the filters in 750mM NaCl, 75mM sodium citrate, 30% formamide, 0.1% sodium dodecyl sulfate, 0.5mg/mL polyvinylpyrrolidone, 0.5mg/mL bovine serum albumin, 0.5mg/mL Ficoll 400, and 0.1mg/mL salmon sperm DNA, at 42°C overnight. The filters were washed in 0.9M NaCl and 90mM sodium citrate at 50°C. A positively hybridizing phage (hkA1-14), was identified and purified by replating and screening with the probe twice more. The final phage plaque was transferred to 0.5 mL 50mM Tris, pH 7.5, 8mM MgSO_4 , 85 mM NaCl, 1mg/mL gelatin, and 1 μL of a 1:50 dilution in water of the phage stock was used as template for PCR amplification. 50 pmol each of 1amL and 1amR (Table I) oligonucleotide primers were included,

- 33 -

then a final 15 min at 72°, according to the GENEAMP protocol (PERKIN ELMER CETUS, Norwalk, CT). A 2.0 kb product was identified by agarose gel electrophoresis, and this was subcloned into the EcoRI site of pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA).
5 Sequence analysis by the SEQUENASE protocol (USBC, Cleveland, OH) demonstrated that this cDNA was homologous to the reported dog A1 clone. SmaI and EcoRI digestion released a DNA fragment containing coding sequence from base pair 76 through the translation STOP codon (Figure 2) that is identical to the human ventricle A1
10 cDNA sequence (clones hval-3a and 5'hval-9). This fragment was used in construction of the full length coding sequence (see below). The human kidney cDNA also includes about 900 bp of 3' untranslated sequence.

15 STEP D:

The human ventricle A2a adenosine receptor partial cDNA (hvA2-13) is a 1.6 kb NotI fragment containing approximately 900 bp 3' untranslated sequence and is 496 bp short of the initiation methionine based on sequence homology to the dog A2a cDNA clone. Two
20 consecutive rounds of 5' RACE were utilized to generate the 5' coding region of the cDNA. First strand cDNA was synthesized from 1 µg of the human ventricle poly(A)⁺ RNA in a total volume of 40 µl containing 50mM Tris, pH 8.0, 140mM KCl, 10mM MgCl₂, 10mM DTT, 15mM each dNTP, 20 units RNasin, 20pmol human primer 68 or
25 74 (for 1st or 2nd round RACE respectively), and 9.2 units AMV reverse transcriptase at 37°C for 2 hrs. The reaction was then diluted to 120µl with 0.5 mM Tris, pH 7.6/0.05 mM EDTA and passed through a SEPHACRYL S-300 SPUN COLUMN. The products in the column effluents were polyadenylated in 100mM potassium cacodylate, pH 7.2, 2 mM CoCl₂, 0.2 mM DTT, 0.15 mM dATP, and 14 units terminal
30 deoxynucleotidyl transferase in a total volume of 31 µl for 10 min. at 37°C. The poly(A) tailing reaction was terminated by heating at 65°C for 15 min. and then diluted to 500 µl with TE.

- 34 -

Five or 10 μ l (for 1st or 2nd round RACE respectively) of the poly(A) tailed first strand cDNA was used as template in the PCR amplification reaction according to the GENEAMP protocol containing 10pmol primer 70, 25 pmol primer 71 (primer 70 and 71 sequences are given above), and 25 pmol human primer 69 or 75 (1st or 2nd round RACE respectively; see Table I) in a total volume of 50 μ l. One cycle of PCR was performed of 1 min at 95°C, 2 min at 50°C, 40 min at 72°C, followed by 40 cycles of 40 sec at 94°C, 2 min at 56°C, 3 min at 72°C. The PCR amplification products were digested with EcoRI and Sall and electrophoresed on a 1.4% agarose gel. Areas corresponding to 200-400 bp were excised and ligated into EcoRI/Sall digested pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA). The sequences of the two A2a PCR products, the 332 bp 1st round RACE product (5'hvA2-14) and the 275 bp 2nd round RACE product (5'hvA2-29) were determined by the SEQUENASE (USBC, Cleveland, OH) protocol. By sequence homology comparisons with the dog A2a adenosine receptor cDNA sequence, the 1st round RACE product (5'hvA2-14) was 258 bp short of the initiation methionine and the second round RACE product (5'HVA2-29) was determined to extend 1bp upstream of the initiation methionine. The human ventricle A2a partial cDNA clone (hvA2-13) and the human A2a PCR products (5'hvA2-14 and 5'hva2-29) contain overlapping sequence and together represent the complete coding sequence for the human adenosine A2a receptor, and include 1 bp and 0.8 kb of 5' and 3' untranslated sequence, respectively. The sequence of the human A2a adenosine receptor is shown in Figures 4A and 4B.

- 35 -

STEP E:

A double-stranded DNA probe was generated by Klenow enzyme extension, including $\alpha^{32}\text{P}$ -dCTP, of annealed oligonucleotides 66 and 67, and used to screen a human striata cDNA library (STRATAGENE, La Jolla, CA). The oligonucleotide sequence was based on a region of the human ventricle A2a cDNA sequence. E. coli strain XL1-blue (STRATAGENE, La Jolla, CA) cells were infected with library phage and grown overnight on agar plates at 37°C. Phage DNA was transferred to HYBOND-N nylon filters according to the manufacturer's protocol (AMERSHAM, Arlington Heights, IL). The probe was incubated with the filters in 750 mM NaCl, 75 mM sodium citrate, 10% formamide, 0.5% sodium dodecyl sulfate, 0.5 mg/mL polyvinylpyrrolidone, 0.5 mg/mL bovine serum albumin, 0.5 mg/mL Ficoll 400, and 0.02 mg/mL salmon sperm DNA, at 42°C overnight. The filters were washed in 0.9 M NaCl and 90 mM sodium citrate at 50°C. A positively hybridizing phage (hbA2-22A) was identified and purified by replating and screening with the probe twice more, and subcloned into the plasmid pBLUESCRIPT SK- by the manufacturer's protocol (STRATAGENE, La Jolla, CA). See Short et al. (1988) Nucl. Acids Res. 16:7583-7600; Sorge (1988) Strategies 1:3-7. The human brain A2a adenosine receptor cDNA (hbA2-22A) spans bp 43 of the A2 coding sequence (Figures 4A and 4B) through the translation STOP codon, and includes about 900 bp of 3' untranslated sequence. The sequence of this human brain A2a cDNA is identical to the human ventricle A2a adenosine receptor cDNA (hvA2-13, 5'hvA2-14 and 5'hvA2-29).

STEP F:

A double-stranded DNA probe was generated by Klenow enzyme extension of annealed oligonucleotides 129 and 130, including $\alpha^{32}\text{P}$ -dCTP, and used to screen a human frontal cortex cDNA library (STRATAGENE, La Jolla, CA). The oligonucleotide sequence was based on a region of the human A2a and A1 cDNA sequence. E. coli strain XL-1 blue (STRATAGENE, La Jolla, CA) cells were infected

- 36 -

transferred to HYBOND-N nylon filters according to the manufacturer's protocol (AMERSHAM, Arlington Heights, IL). The probe was incubated with the filters in 750 mM NaCl, 75 mM sodium citrate, 10% formamide, 0.5% sodium dodecyl sulfate, 0.5 mg/mL polyvinyl-pyrrolidone, 0.5 mg/mL bovine serum albumin, 0.5 mg/mL Ficoll 400, and 0.02 mg/mL salmon sperm DNA, at 42°C overnight. The filters were washed in 0.9 M NaCl and 90 mM sodium citrate at 50°C. A positively hybridizing phage (hb-32c), was identified and purified by replating and screening with the probe twice more. The insert was subcloned to the plasmid pBLUESCRIPT SK- according to the manufacturer's protocol (STRATAGENE, La Jolla, CA). Sequence analysis by the SEQUENASE protocol (USBC, Cleveland, OH) demonstrated a complete open reading frame coding for amino acid sequence homologous to both of the previously isolated human A1 and A2a clones. This homologous adenosine receptor subtype cDNA is the A2b subtype having the sequences in Figures 5 and 6. A 1.3 kb SmaI-XmnI fragment was ligated into the SmaI site of pSVL (PHARMACIA, Piscataway, NJ), giving the full length coding sequence of the A2b adenosine receptor in a plasmid suitable for its expression in COS and CHO cells. See Sprague et al. (1983) J. Virology 45:773; Templeton and Eckhart (1984) Mol. Cell Biol. 4:817.

Table I:

Sequences and directions of the primers used in the isolation of cDNA's and construction of expression plasmids, along with the positions in the clones upon which the sequences are based. Dog A1 and A2a cDNA clones are from F. Libert, et al, (1989) Science 244:569-572. Primers LamL and LamR are based on the sequence of λ gt10 (T.V. Hyunh, et al. (1985) DNA Cloning: A Practical Approach, Vol 1, D. Glover, ed, IRL Press, Oxford). The A2b adenosine receptor subtype encoded by the clone hb32C was determined to be the A2b adenosine receptor subtype on the basis of the binding profile of the adenosine receptor agonist NECA and affinities for adenosine receptor

- 37 -

antagonists measured on membranes prepared from pSVLhb32C
transfected COS7, CHO or HEK 293 cells.

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- 38 -

	name	sequence	position	clone	direction
5	52	ATTCGCAGCCACGTCCTGA- GGCGGCGGGAGCCCTTCAA- AGCAGGTGGCACCAGTGCC- CGC (SEQ ID NO. 1)	1201-1260	dog A2a	sense
10	53	GCGGAGGCTGATCTGCT- CTCCATCACTGCCATGAG- CTGCCAAGGCGCGGGCAC- TGGTGCC (SEQ. ID NO. 2)	1305-1246	dog A2a	antisense
15	62	TCCAGAAGTTCCGGGTCA- CCTTCCTTAAGATCTGGAA- TGACCACTTCCGCTGCCAGC- CCA (SEQ. ID NO. 3)	958-1017	dog A1	sense
20	63	AGTCGTGGGGCGCCTCCT- CTGGGGGGGTCCTCGTCGAC- GGGGGGCGTGGGCTGGCAG- CGGA (SEQ ID NO. 4)	1062-1003	dog A1	antisense
25	66	GCCTCTTTGAGGATGTGG- TCCCCATGAACTACATGGT- GTACTTCA (SEQ ID NO. 5)	500-542	5'hvA2-14	sense
30	67	GCAGGGGCACCAGCACACA- GGCAAAGAAGTTGAAGTAC- ACCATGT (SEQ ID NO. 6)	572-528	5'hva2-14	antisense

- 39 -

	name	sequence	position	clone	direction
5	68	TCGCGCCGCCAGGAAGAT (SEQ ID NO 7)	616-599	hva2-13	antisense
	69	TATATTGAATTCTAGACAC- CCAGCATGAGC (SEQ ID NO.8)	591-574	hva2-13	antisense
10	74	TCAATGGCGATGGCCAGG (SEQ ID NO. 9)	303-286	5'hva2-14	antisense
15	75	TATATTGAATTCATGGA- GCTCTGCGTGAGG- (SEQ ID NO. 10)	276-259	5'hva2-14	antisense
20	79	GTAGACCATGTACTCCAT (SEQ ID NO. 11)	560-543	hva1-3a	antisense
	80	TATATTGAATTCTGACCT- TCTCGAACTCGC- (SEQ ID NO. 12)	537-521	hva1-3a	antisense
25	81	ATTGAATTCGATCACGGG- CTCCCCCATGC- (SEQ ID NO. 13)	515-496	hva1-3a	antisense
30	129	ATGGAGTACATGGTCTAC- TTCAACTTCTTTGTGTGGG- TGCTGCCCCCGCT- (SEQ ID NO. 14)	---	---	sense

- 40 -

	name	sequence	position	clone	direction

5	130	GAAGATCCGCAAATAGACA- CCCAGCATGAGCAGAAGCG- GGGGCAGCACCC (SEQ ID NO. 15)	---	---	antisense
10	131	CCCTCTAGAGCCCAGCCTGT- GCCCGCCATGCCCATCATGG- GCTCC (SEQ ID NO. 16)	2-19 1-14	5'hva2-29 5'hva1-9	sense
15	lamL	CCCACCTTTTGAGCAAGTTC (SEQ ID NO. 17)	---	λ t10	---
	lamR	GGCTTATGAGTATTTCTTCC (SEQ ID NO. 18)	---	λ t10	---
20	207	CCCAAGCTTATGAAAGCCAA CAATACC (SEQ ID NO. 27)			
25	208	TGCTCTAGACTCTGGTATCT TCACATT (SEQ ID NO. 28)			

EXAMPLE 2

- 30 Human A1 adenosine receptor expression construct:
- To express the human adenosine receptor cDNA in COS, CHO and HEK 293 cells, the 118bp Sall-SmaI fragment of the human ventricle A1 PCR product (5'HVA1-9) was ligated together with the 1.8 SmaI-EcoRI fragment of the human kidney A1 adenosine receptor cDNA (hkA1-14) and the 3.0 kb Sall-EcoRI fragment of

- 41 -

pBLUESCRIPT II KS+, resulting in a plasmid containing the contiguous full length coding sequence for the human A1 adenosine receptor cDNA and some 5' and 3' untranslated sequence. This plasmid was digested first with EcoRI, the resulting ends were filled in by Klenow enzyme
5 extension and then the plasmid was digested with XhoI to release a fragment of 1.9 kb containing the full length human A1 adenosine receptor cDNA. The fragment was subcloned into the expression vector pSVL (PHARMACIA) which had been digested with XhoI-SmaI.

10 Human A2a adenosine receptor expression construct:

To express the human A2a adenosine receptor cDNA in COS, CHO or HEK 293 cells, a contiguous A2a cDNA sequence was constructed before subcloning into the expression vector, pSVL. Primer 131, containing an XbaI recognition site, 14 bp of 5'
15 untranslated sequence of human A1 adenosine receptor cDNA, and the first 18 bp of human A2a adenosine receptor cDNA coding sequence was used with primer 75 in PCR with 1 ng of the plasmid containing the human ventricle A2a 2nd round RACE product (5'hvA2-29) as
20 template. Twenty-five cycles of 40 sec at 94°C, 1 min at 55°C, and 3 min at 72°C, then a final incubation of 15 min at 72°C, with 1 ng of plasmid template and 50 pmol of each primer in a volume of 50 µL according to the GENEAMP protocol (PERKIN ELMER CETUS,
Norwalk, CT), resulted in the expected 302 bp product determined by
25 agarose gel electrophoresis. The 172 bp XbaI-EagI digestion product of this DNA fragment was ligated together with 1125 bp EagI-BglII digestion product of the human striata A2a adenosine receptor cDNA (hbA2-22A) and XbaI-SmaI digested pSVL (PHARMACIA), generating the full length human A2a adenosine receptor cDNA coding sequence in
30 a plasmid suitable for its expression in COS, CHO or HEK 293 cells.

Mammalian cell expression:

COS7 cells (ATCC #1651-CRL) were grown in complete medium, Dulbecco's modified Eagles's medium, DMEM (GIBCO, Grand Island, NY) containing 10% fetal bovine serum, 100U/mL

- 42 -

penicillin-streptomycin and 2 mM glutamine, in 5% CO₂ at 37°C. Transient transfection of COS7 cells was performed by the CaPO₄ method (Graham, F.L. and Van Der Erb, A.J. (1973) Virology 52:456-567) using the Mammalian Transfection Kit (STRATAGENE). See
5 Chen and Okayama Mol. Cell Biol. 7:2745-2752. Plasmid DNA (15 µg) was precipitated with 125 mM CaCl₂ in BBS (N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid buffered saline) at room temperature for 30 minutes. The DNA precipitate was added to the COS7 cells and incubated for 18h in 5% CO₂ at 37°C. The precipitate was removed
10 and the cells were washed twice with serum free DMEM. Cells were incubated in complete medium in 5% CO₂ at 37°C for 48 h prior to the binding assay.

Stable expression in CHO or HEK 293 cells:

15 To establish stable cell lines, CHO or HEK 293 cells were co-transfected with 20 µg of pSVL containing the adenosine receptor cDNA and 1 µg of pWLneo (STRATAGENE) containing the neomycin gene. See Southern and Berg (1982) J. Mol. App. Gen. 1:327-341. Transfection was performed by the CaPO₄ method. DNA was
20 precipitated at room temperature for 30 minutes, added to the CHO cells and incubated 18h in 5% CO₂ at 37°C. The precipitate was removed and the cells were washed twice with serum free DMEM. Cells were incubated for 24h in 5% CO₂ at 37°C, replated in 24-well
25 dishes at a dilution of 1:10, and incubated an additional 24h before adding selection medium, DMEM containing 10% fetal bovine serum, 100U/mL penicillin-streptomycin, 2 mM glutamine and 0.5 mg/mL G418 (GIBCO). Transfected cells were incubated at 5% CO₂, 37°C until viable colonies were visible, approximately 14-21 days. Colonies were selected and propagated. The cell clone with the highest number
30 of human adenosine receptors was selected for subsequent application in the binding assay.

- 43 -

EXAMPLE 3Binding studies:

5 Membranes were prepared from transiently transfected
COS7 cells 48 h after transfection or from G418-selected stably
transfected CHO or HEK 293 cells. Cells were harvested in 1 mM
EDTA in phosphate buffered saline and centrifuged at 2000 x g for 10
minutes. The cell pellet was washed once with phosphate buffered
10 saline. The cell pellet was resuspended in 2 mL of 5 mM Tris, pH 7.6/
5mM MgCl₂. Membranes were prepared from the cells by freeze-thaw
lysis in which the suspension was frozen in a dry ice/ethanol bath and
thawed at 25°C twice. The suspension was homogenized after adding an
additional 2 mL of 5 mM Tris, pH 7.6/5 mM MgCl₂, in a glass dounce
15 homogenizer with 20 strokes. The membranes were pelleted at 40,000
x g at 4°C for 20 minutes. The membrane pellet was resuspended at a
protein concentration of 1-2 mg/mL in binding assay buffer, 50 mM
Tris, pH 7.6/10 mM MgCl₂. Protein concentration was determined by
the method of Bradford ((1976) Anal. Biochem. 72: 248-250). Before
the binding assay was performed, the membranes were incubated with
20 adenosine deaminase (BOEHRINGER MANNHEIM), 2 U/mL for 30
minutes at 37°C. Saturation binding of [³H]-cyclohexyladenosine (CHA)
was performed on membranes prepared from pSVLA1 transfected
COS7 or CHO cells.

 Membranes (100µg) were incubated in the presence of 0.2
25 U/mL adenosine deaminase with increasing concentrations of CHA
(NEN, 32 Ci/mmol) in the range of 0.62 - 30 nM for 120 minutes at
25°C in a total volume of 500 µL. The binding assay was terminated by
rapid filtration and three washes with ice-cold 50 mM Tris, pH 7.6/10
mM MgCl₂ on a SKATRON CELL HARVESTER equipped with a
30 receptor binding filtermat (SKATRON INSTRUMENTS, INC). Non-
specific binding was determined in the presence of 100 µM N⁶-
cyclopentyladenosine (CPA). Bound radioactivity was measured by
scintillation counting in READY SAFE SCINTILLATION COCKTAIL
(BECKMAN). For competition binding experiments, membranes were

- 44 -

incubated with 5 nM [^3H]-CHA and various concentrations of A1 adenosine receptor agonists. Saturation binding of [^3H] CGS-21680 was performed on membranes prepared from pSVLA2a transfected COS7 cells. Membranes (100 μg) were incubated in the presence of 0.2 U/mL adenosine deaminase with increasing concentrations of CGS21680 (NEN, 48.6 Ci/mmol) in the range of 0.62 -80 nM for 90 minutes at 25°C in a total volume of 500 μL . The binding assay was terminated by rapid filtration with three washes with ice-cold 50 mM Tris, pH 7.6/10 mM MgCl_2 on a Skatron cell harvester equipped with a receptor binding filtermat (SKATRON INSTRUMENTS, INC). Non-specific binding was determined in the presence of 100 μM CPA. Bound radioactivity was measured by scintillation counting in READY SAFE LIQUID SCINTILLATION COCKTAIL (BECKMAN). For competition binding experiments, membranes were incubated with 5nM [^3H]-CGS21680 and various concentrations of A2 adenosine receptor agonists.

Saturation binding of [^3H]5'-N-ethylcarboxamidoadenosine (NECA) was performed on membranes (100 μg) prepared from pSVLhb32C (A2b) transfected COS7 cells in the presence of adenosine deaminase with increasing concentrations of NECA (NEN, 15.1Ci/mmol) in the range of 1.3-106 nM for 90 minutes at 25°C in a total volume of 500 μL . The assay was terminated by rapid filtration and three washes with ice-cold binding buffer on a cell harvester equipped with a receptor binding filtermat (SKATRON INSTRUMENTS, INC). Bound radioactivity was measured by scintillation counting. Non-specific binding was measured on membranes prepared from non-transfected COS7 cells. For competition binding experiments, membranes from transfected cells were incubated with 10 nM [^3H]NECA and varying concentrations of adenosine receptor antagonists.

- 45 -

EXAMPLE 4

The human A3 adenosine receptor was cloned from a human striata cDNA library. Oligonucleotide probes were designed based on the rat A3 sequence of Zhou et al., Proc. Natl. Acad. Sci. 89, 7432 (1992). The complete sequence of the human A3 adenosine receptor was determined and the protein sequence deduced. The cloned human A3 adenosine receptor is expressed in a heterologous expression system in COS, CHO and HEK 293 cells. Radiolabeled adenosine receptor agonists and antagonists are used to measure the binding properties of the expressed receptor. Stable cell lines can be used to evaluate and identify adenosine receptor agonists, antagonists and enhancers.

STEP A:

A synthetic probe homologous to the rat A3 adenosine receptor was generated using the polymerase chain reaction (PCR). Three μ l of rat brain cDNA was used as template in a PCR amplification reaction according to the GENEAMP protocol (PERKIN ELMER CETUS, Norwalk, CT) containing 50 pmol of primers 207 (5'-cccaagcttatgaaagccaacaatacc) (SEQ. ID NO: 27) and 208 (5'-tgctctagactctggtatcttcacatt) (SEQ. ID NO: 28) in a total volume of 50 μ l. Primers 207 and 208 are based on the published rat A3 adenosine receptor sequence (Zhou, et al, (1992), Proc. Natl. Acad. Sci. USA, 89:7432-7406). Forty cycles of 40 sec at 94°C, 1 min at 55°C, 3 min at 72°C were performed and the resulting 788 bp fragment was subcloned into HindIII-XbaI digested pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA). The sequence was verified by the SEQUENASE protocol (USBC, Cleveland, OH).

STEP B:

The 788 bp PCR fragment was labeled with $\alpha^{32}\text{P}$ -dCTP using the MULTIPRIME DNA LABELLING SYSTEM (AMERSHAM, Arlington Heights, IL) and used to screen a human striata cDNA library

- 46 -

(STRATAGENE, La Jolla, CA). *E. coli* strain XL-1 Blue (STRATAGENE, La Jolla, CA) cells were infected with library phage and grown overnight at 37°C. Phage DNA was transferred to HYBOND-N nylon filters according to the manufacturer's protocol (AMERSHAM, Arlington Heights, IL). The probe was incubated with the filters in 5 X SSC, 30% formamide, 5 X Denhardt's solution, 0.5% sodium dodecyl sulfate, and 50 µg/ml sonicated salmon testis DNA. The filters were washed in 2 X SSC at 55°C. A positively hybridizing phage (HS-21a) was identified and plaque purified by two additional rounds of plating and hybridization. The insert was subcloned to the plasmid pBLUESCRIPT II SK- according to the manufacturer's protocol (STRATAGENE, La Jolla, CA). Upon sequence analysis using the SEQUENASE protocol (USBC, Cleveland, OH) it was determined that clone HS-21a contained the complete open reading frame corresponding to the human homolog of the rat A3 adenosine receptor. The coding region of the human A3 adenosine receptor cDNA is 78% identical to the rat sequence at the nucleotide level and contains 265 bp and 517 bp of 5' and 3' untranslated sequence, respectively. The 1.7 kb fragment was excised using sites present in the multiple cloning site of pBLUESCRIPT II SK- (STRATAGENE, La Jolla, CA) and subcloned into XhoI/SacI digested pSVL (PHARMACIA, Piscataway, NJ) for its expression in COS and CHO cells.

EXAMPLE 5

Mammalian cell expression:

COS7 cells (ATCC #1651-CRL) were grown in complete medium, Dulbecco's modified Eagles's medium, DMEM (GIBCO, Grand Island, NY) containing 10% fetal bovine serum, 100U/mL penicillin-streptomycin and 2mM glutamine, in 5% CO₂ at 37°C. Transient transfection of COS7 cells was performed by the CaPO₄ method (Graham, F.L. and Van Der Erb, A.J. (1973) Virology 52:456-567) using the Mammalian Transfection Kit (STRATAGENE). Plasmid DNA (15 µg) was precipitated with 125 mM CaCl₂ in BBS (N,N-bis(2-

- 47 -

hydroxyethyl)-2-aminoethanesulfonic acid buffered saline) at room temperature for 30 minutes. The DNA precipitate was added to the COS7 cells and incubated for 18 h in 5% CO₂ at 37°C. The precipitate was removed and the cells were washed twice with serum free DMEM.
5 Cells were incubated in complete medium in 5% CO₂ at 37°C for 48 h prior to the binding assay.

Stable expression in CHO cells:

To establish stable cell lines, CHO cells were cotransfected
10 with 20 µg of pSVL containing the adenosine receptor cDNA and 1 µg of pWLneo (STRATAGENE) containing the neomycin gene. Transfection was performed by the CaPO₄ method. DNA was precipitated at room temperature for 30 minutes, added to the COS7 cells and incubated 18 h in 5% CO₂ at 37°C. The precipitate was
15 removed and the cells were washed twice with serum free DMEM. Cells were incubated for 24 h in 5% CO₂ at 37°C, replated in 24-well dishes at a dilution of 1:10, and incubated an additional 24 h before adding selection medium, DMEM containing 10% fetal bovine serum, 100U/mL penicillin-streptomycin, 2 mM glutamine and 1.0 mg/mL
20 G418 (GIBCO). Transfected cells were incubated at 5% CO₂, 37°C until viable colonies were visible, approximately 14-21 days. Colonies were selected and propagated. The cell clone with the highest number of human adenosine receptors was selected for subsequent application in the binding assay.

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EXAMPLE 6

Binding assay:

Membranes were prepared from transiently transfected
30 COS7 cells 48 h after transfection or from G418-selected stably transfected CHO or HEK 293 cells. Cells were harvested in 1 mM EDTA in phosphate buffered saline and centrifuged at 2000 x g for 10 minutes. The cell pellet was washed once with phosphate buffered saline. The cell pellet was resuspended in 2 mL of 5 mM Tris, pH 7.6/

- 48 -

5mM MgCl₂. Membranes were prepared from the cells by freeze-thaw lysis in which the suspension was frozen in a dry ice/ethanol bath and thawed at 25°C twice. The suspension was homogenized after adding an additional 2 mL of 5 mM Tris, pH 7.6/ 5mM MgCl₂, in a glass dounce homogenizer with 20 strokes. The membranes were pelleted at 40,000 x g at 4°C for 20 minutes. The membrane pellet was resuspended at a protein concentration of 1-2 mg/mL in binding assay buffer, 50 mM Tris, pH 7.6/10 mM MgCl₂. Protein concentration was determined by the method of Bradford ((1976) Anal. Biochem. 72: 248-250). Before the binding assay was performed, the membranes were incubated with adenosine deaminase (BOEHRINGER MANNHEIM), 2U/mL for 30 minutes at 37°C. Saturation binding of [¹²⁵I]-N⁶-aminobenzyl-adenosine (125I-ABA) or [¹²⁵I]-N⁶-2-(4-amino-3-iodophenyl)ethyl-adenosine (APNEA) was performed on membranes prepared from pSVLA3 transfected COS7 cells. Membranes (100 µg) were incubated in the presence of 0.2U/mL adenosine deaminase with increasing concentrations of 125I-ABA in the range of 0.1-30 nM for 120 minutes at 25°C in a total volume of 500 µL. The binding assay was terminated by rapid filtration and three washes with ice-cold 50 mM Tris, pH 7.6/10 mM MgCl₂ on a Skatron cell harvester equipped with a receptor binding filtermat (SKATRON INSTRUMENTS, INC). Non-specific binding was determined on non-transfected cells. Bound radioactivity was measured by scintillation counting in Ready Safe Scintillation Cocktail (BECKMAN).

EXAMPLE 7

In vitro transcription and oocyte expression:

30 The 1.3 kb XhoI-BamHI fragment of the pSVL expression construct (described in Example 2) containing the full length human A2a adenosine receptor coding sequence was ligated into SaII-SpeI digested pGEMA (Swanson, et al, (1990) Neuron 4:929-939). The resulting plasmid, pGEMA2, was linearized with NotI, forming a template for in vitro transcription with T7 RNA polymerase. The

- 49 -

homologous adenosine receptor subtype cDNA in pBluescript SK- was used as a template for in vitro transcription by T3 polymerase after removal of most of the 5' untranslated region, with the exception of 20 bp, as a 0.3 kb SmaI fragment. The K⁺ channel cDNA, Kv3.2b was employed as a negative control in the cAMP accumulation assay. The generation of Kv3.2b RNA was described by Luneau, et al, ((1991) FEBS Letters 1:163-167). Linearized plasmid templates were used with the STRATAGENE mCAP kit according to the manufacturer's protocol, except that the SP6 RNA polymerase reaction was performed at 40°C. Oocytes were harvested from mature female *Xenopus laevis*, treated with collagenase, and maintained at 18°C in ND96 medium (GIBCO) supplemented with 1 mM sodium pyruvate and 100 µg/mL gentamycin. Fifty nanoliters (10 ng) of RNA diluted in H₂O was injected and oocytes were incubated at 18°C for 48 hours.

EXAMPLE 8

cAMP accumulation assay in oocytes:

Oocytes injected with either human adenosine receptor transcript or the Kv3.2b transcript were transferred to fresh medium supplemented with 1 mM of the phosphodiesterase inhibitor, Ro 20-1724 (RBI, Natick, MA) and 1 mg/mL bovine serum albumin incubated for 30 minutes and transferred to an identical medium with or without the agonist adenosine (10 mM) for an additional 30 minutes at room temperature. Groups of 5-10 oocytes were lysed by transfer to ND96/100 mM HCl/1 mM Ro 20-1724 in microfuge tubes, shaken, incubated at 95°C for 3 min, and centrifuged at 12000 g for 5 min. Supernatants were stored at -70°C before cAMP measurements. Cyclic AMP levels were determined by radioimmunoassay (RIANEN kit, DuPont/NEN) using the acetylation protocol. The adenosine receptor antagonist, 8-(p-sulfophenyl)theophylline (100 µM) was utilized to inhibit the cAMP response induced by adenosine in oocytes expressing the adenosine receptors.

- 50 -

EXAMPLE 9

cAMP accumulation in stable CHO cell lines:

5 The changes in cAMP accumulation can alternatively be measured in stably transfected CHO cells expressing the human adenosine receptor subtypes. CHO cells are washed twice in phosphate buffered saline (PBS) and detached in 0.2% EDTA in PBS. The cells are pelleted at 800 rpm for 10 min and resuspended in KRH buffer (140 mM NaCl/5 mM KCl/2 mM CaCl₂/1.2 mM MgSO₄/1.2 mM KH₂PO₄/6 mM glucose/25 mM Hepes buffer, pH 7.4). The cells are washed once
10 in KRH buffer and resuspended at 10⁷ cells/mL. The cell suspension (100 µL) is mixed with 100 µL of KRH buffer containing 200 µM Ro 20-1724 and incubated at 37°C for 10 minutes. Adenosine (10 µM) was added in 200 µL KRH buffer containing 200 µM Ro 20-1724 and
15 incubated at 37°C for 20 minutes. After the incubation, 400 µL of 0.5 mM NaOAc (pH 6.2) was added and the sample was boiled for 20 minutes. The supernatant was recovered by centrifugation for 15 minutes and stored at -70°C. cAMP levels were determined by radioimmunoassay (RIANEN kit, DuPont/NEN) using the acetylation
20 protocol. The effect of antagonists on cAMP accumulation are measured by preincubation for 20 minutes before adding adenosine.

EXAMPLE 10

Expression Construct and Transfection

25 The 1.7 kb HS-21a cDNA (A3) was subcloned as a Sall-BamHI fragment into the expression vector pCMV5 (Mumby, S.M., Heukeroth, R.O., Gordon, J.I. and Gilman, A.G. (1990) Proc. Natl. Acad. Sci. USA **87**, 728-732.) creating the vector pCMV5-A3. CHO or HEK 293 cells stably expressing the human HS-21a cDNA were
30 prepared by co-transfection of 15 µg pCMV5-A3 and 1 µg pWLneo (Stratagene) using the calcium phosphate method. Stable cell lines were also generated using EBV based mammalian expression vectors, pREP (INVITROGEN). Neomycin resistant colonies were selected in 1

- 51 -

mg/mL G418 (GIBCO). Stable colonies were screened for expression of HS-21a by ^{125}I -ABA binding.

EXAMPLE 11

5 Binding Studies

Membranes were prepared from stable CHO cell lines in 10 mM Hepes, pH 7.4 containing 0.1 mM benzamidine and 0.1 mM PMSF as described (Mahan, L.C., et al., (1991) Mol. Pharmacol. **40**, 1-7). Pellets were resuspended in 5 mM Hepes, pH 7.4/5 mM MgCl_2 /0.1
10 mM benzamidine/0.1 mM PMSF at a protein concentration of 1-2 mg/mL and were incubated with adenosine deaminase (Boehringer Mannheim), 2U/mL at 37 °C for 20 minutes. Saturation binding of ^{125}I -ABA was carried out on 50 μg of membranes for 120 minutes at 25 °C in a total volume of 100 μL . The assay was terminated by rapid
15 filtration and three washes with ice-cold binding buffer on a Skatron harvester equipped with a receptor binding filtermat (Skatron Instruments, INC). The specific activity of ^{125}I -ABA, initially 2,200 Ci/mmol, was reduced to 100 Ci/mmol with nonradioactive I-ABA for saturation analysis. Nonspecific binding was measured in the presence of
20 1 μM I-ABA. The K_D and B_{max} values were calculated by the EBDA program (McPherson, G.A. (1983) Computer Programs for Biomedicine **17**, 107-114). Competition binding of agonists and antagonists was determined with ^{125}I -ABA (0.17-2.0 nM, 2000 Ci/mmol). Nonspecific binding was measured in the presence of 400 μM
25 NECA. Binding data were analyzed and competition curves were constructed by use of the nonlinear regression curve fitting program Graph PAD InPlot, Version 3.0 (Graph Pad Software, San Diego). K_i values were calculated using the Cheng-Prusoff derivation (Cheng, Y.C. and Prusoff, H.R. (1973) Biochem. Pharmacol. **22**, 3099-3108.).

30 The binding properties of the receptor encoded by HS-21a were evaluated on membranes prepared from CHO cells stably expressing the HS-21a cDNA. The radioligand, ^{125}I -APNEA, was previously used to characterize rat A3 adenosine receptors. In preliminary experiments, high non-specific ^{125}I -APNEA binding to

- 52 -

CHO cell membranes was observed which interfered with the measurement of specific binding to expressed receptors. Specific and saturable binding of the adenosine receptor agonist, ^{125}I -ABA was measured on membranes prepared from the stably transfected cells (Figure 11A). The specific binding of ^{125}I -ABA could be prevented by either 1 mM nonradioactive I-ABA or 400 μM NECA. No specific binding of ^{125}I -ABA was measured on membranes prepared from non-transfected CHO cells. The specific binding of ^{125}I -ABA measured in either the presence of 10 μM GTP γ S or 100 mM Gpp(NH)p was reduced by 56 and 44% respectively, relative to the specific binding measured in the absence of the uncoupling reagents. These results suggest that ^{125}I -ABA exhibits some agonist activity on the receptor encoded by the HS-21a cDNA expressed in the stable CHO cell line. ^{125}I -ABA binds to membranes prepared from the HS-21a stable CHO cells with a dissociation constant of 10 nM ($B_{\text{max}} = 258 \text{ fmol/mg}$ protein) with a Hill coefficient of 0.99 indicating binding to a single class of high affinity sites (Figure 11B).

The competition of adenosine receptor agonists and antagonists for binding to HS-21a receptors was determined (Figures 12A and 12B). The K_i values for agonists (top panel) were calculated to be 26 nM for NECA, 34 nM for R-PIA, 89 nM for CPA and 320 nM for S-PIA, resulting in a potency order profile of NECA > R-PIA > CPA > S-PIA. In contrast to the insensitivity of adenosine receptor antagonists reported for the rat A3 adenosine receptor subtype, a number of xanthine antagonists exhibited competition with ^{125}I -ABA for binding to the HS-21a receptor (lower panel). Studies of the sheep A3 adenosine receptor indicated that 8-phenylxanthines substituted in the para-position with acidic substituents are high affinity antagonists. By evaluating additional xanthines in this class we determined that I-ABOPX is the highest affinity antagonist yet reported for A3 adenosine receptors. The K_i values for antagonists were calculated to be 18 nM for I-ABOPX, 55 nM for BW-A1433, 70 nM for XAC and 750 nM for DPCPX, resulting in a potency order profile of I-ABOPX > BW-A1433 > XAC > DPCPX.

- 53 -

EXAMPLE 12cAMP Studies

5 Determinations were made on stably transfected CHO cells in suspension as described (Linden et al., (1993) Mol. Pharm. 44:524-532). Supernatants (500 μ L) were acetylated and acetylcyclic AMP was measured by automated radioimmunoassay (Hamilton, B.R. and Smith, D.O. (1991) J. Physiol. (Lond.) 432, 327-341). Antagonist dissociation constants were estimated from pA_2 values as described by Schild (1957) Pharm. Rev. 9, 242-246).

10 The ability of the HS-21a receptor stably expressed in CHO cells to couple to the cAMP regulatory system was measured using adenosine as an agonist. Adenosine (10 μ M) produced a 30 % inhibition of the forskolin-stimulated increase in cAMP. In the absence of
15 forskolin, adenosine had no effect on the cAMP levels. In non-transfected CHO cells, adenosine had no effect on cAMP levels when measured with or without forskolin treatment.

I-ABA produced only about half as much inhibition of
20 forskolin-stimulated cyclic AMP accumulation in CHO cells as did NECA and other agonists (PIA and CPA). Furthermore, in the presence of I-ABA, the dose response curve of NECA to lower cyclic AMP was right-shifted. These data indicate that I-ABA is a partial agonist in this system. Dose-response curves of NECA-induced inhibition of forskolin-stimulated cAMP accumulation were also right shifted in the presence of
25 competing xanthine antagonists (Figures 13A through 13F). Schild analyses were used to estimate the K_i from pA_2 values. The K_i values determined by competitive binding for various agonists and antagonists are compared with the K_A values in the functional cAMP assay in Table 1. The potency order profiles were nearly identical for the binding and
30 functional assays, however, the K_a of agonists to lower cAMP were consistently higher (i.e. lower potency) than K_i values determined from competitive binding assays. Although the conditions of these assays differ, these data suggest that recombinant A3 receptors are not well coupled to inhibition of cyclic AMP accumulation in CHO cells.

- 54 -

EXAMPLE 13

Northern Blot Analysis

Human poly(A)⁺ RNA from different tissue sources (Clontech) was fractionated on a 1% agarose-formaldehyde gel (Sambrook, J., Fritsch, E. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Press, Cold Spring Harbor, NY), transferred to Hybond-N membranes and hybridized in 5XSSPE, 5XDenhardt's, 0.5% SDS, 50 mg/mL sonicated salmon testis DNA, with 30% formamide (for A1, A2a, and A2b) or 50% formamide (for HS-21a) at 42°C. DNA probes corresponding to nucleotides 512-1614, 936-2168, and 321-1540 of accession numbers X68485(A1), X68486(A2a), and X68487(A2b) respectively, and a 1.7 kb Sall-BamHI fragment of HS-21a were labeled with a³²P-dCTP by the random priming method. Filters were washed under high stringency conditions in 0.1XSSC at 65°C.

Poly A⁺ RNA from a number of human tissues was evaluated by Northern blot analyses to establish the distribution of tissue expression for the HS-21a transcript (Figure 14A). A 2 kb transcript was most abundantly expressed in lung and liver, with moderate amounts observed in brain and aorta. Low levels of expression were also observed in testis and heart. No expression was detected in spleen or kidney. The profile of lung=liver>>brain=aorta>testis>heart determined for HS-21a is considerably different from the tissue distribution of the other human adenosine receptor subtypes (Figure 14B). A human A1 transcript (2.9 kb) is expressed in brain, heart, kidney and lung with the most abundant expression observed in the brain. A second hybridizing band of 4.3 kb is also observed in lower amounts in the brain. In contrast to the results obtained for the A1 adenosine receptor, the A2a adenosine receptor transcript (2.8 kb) is equally expressed in brain, heart and kidney with slightly higher levels of expression detected in the lung. (Two hybridizing bands were observed when the full length A2a coding sequence was used as a probe and may be the result of cross-hybridization with the A1 transcript

- 55 -

(upper band)). In human brain, the expression of the A1 adenosine receptor subtype is most abundant in the cortex (25) and the expression of the A2a adenosine receptor subtype mRNA has been shown by in situ hybridization to be restricted to the caudate, putamen and nucleus accumbens (26). The human brain mRNA utilized in the Northern analysis was prepared from the brain stem, pons, cerebellum, telencephalon, diencephalon and mesencephalon regions of the brain and does not represent enriched transcripts from those regions of the brain in which the most abundant expression of A1 and A2a adenosine receptors has been indicated by radioligand binding or in situ hybridization studies. For the human A2b subtype, two hybridizing transcripts of 1.7kb and 2.1kb were observed in brain, heart and lung. The smaller 1.7kb transcript was more abundant. In contrast to the expression of the A1 and A2a adenosine receptor transcripts, no expression of A2b transcript was observed in the kidney. From the comparison of the distribution of human adenosine receptor transcripts, it can be concluded that the subtype transcripts are widely distributed but differ from each other in the abundance found in particular tissues.

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EXAMPLE 14

SPECIFIC INHIBITION OF ADENOSINE INDUCED VASCULAR CONSTRICTION AND SPECIFIC INHIBITION OF ADENOSINE INDUCED MAST CELL DEGRANULATION

We have discovered that adenosine, adenosine metabolites and other A3 adenosine receptor agonists induce mast cell degranulation in an animal model and that this can be prevented by selective antagonists of the A3 receptor. The release of enzymes, bioactive amines and arachidonic acid metabolites following mast cell activation causes vasoconstriction, edema, leukocyte accumulation, and ultimately, tissue damage. Mast cell degranulation is a component of: myocardial reperfusion injury, hypersensitivity reactions (asthma, allergic rhinitis, and urticaria), ischemic bowel disease, autoimmune inflammation, and atopic dermatitis. The invention consists of the use of any of a series of highly specific A3 adenosine receptor antagonists to treat or prevent

- 56 -

these diseases and pathologic effects that result from mast cell degranulation.

Adenosine is a potent vasodilator that has also been shown to cause vasoconstriction. The constrictor response has classically been attributed to A1 adenosine receptor stimulation or interactions with the renin-angiotensin system. A previously unreported vasoconstrictor action of adenosine in hamster cheek pouch arterioles is described here, and the specific blockade of this response by A3 adenosine receptor antagonists is demonstrated. Adenosine, inosine, cromolyn, compound 48/80, methylene blue, acetylcholine, and components for saline solutions used to bathe arterioles were obtained from Sigma. 8(p-sulphophenyl)theophylline was obtained from Research Biochemicals, Inc (Natick, MA).

Arterioles (luminal diameter approximately 60 μm) were dissected from male Golden hamster cheek pouches, transferred to a 37°C tissue chamber, and cannulated at both ends (see Duling et al., Am. J. Physiol. 241 (Heart Circ. Physiol. 10): H108-H116, 1981; Duling et al., Microcirculatory Technology, edited by C.H. Baker and W.G. Nastuk, Orlando Academic Press, 1986, p.265-280). Changes in luminal diameter in response to abluminal delivery of adenosine (10^{-8} M to 10^{-4} M) were measured using videotaped microscopic observation and video calipers with continuous output, to generate cumulative concentration-response curves. These curves were discovered to be biphasic: 10^{-6} M adenosine elicited an intense, transient constriction and higher concentrations induced dilator responses. Pretreatment (100 μM) with 8(p-sulfophenyl) theophylline, SPT, a nonspecific adenosine receptor antagonist, inhibited the dilator responses but did not alter the constriction. Without more, this result is consistent with the interpretation that the constrictor response is not mediated through an adenosine A1 or A2 receptor.

However, we made the critical observation that the constrictor response was assymetrical and focal in nature, such that it was initiated at discrete points and subsequently spread along the entire vessel, suggesting discrete sites of action of adenosine. Examination of

- 57 -

the abluminal surface of the vessel after staining with methylene blue revealed large numbers of mast cells closely associated with the vessel wall (figure 16). Following exposure to adenosine, mast cells were found to be degranulated, suggesting the involvement of mast cell granule contents in the constrictor response. This finding was consistent with reports that adenosine potentiates the release of granule contents from mast cells isolated from rat peritoneum (Lohse et al., N.-S. Arch. Pharmacol. 335:555-560, 1987; Marquardt et al., J. Immunol. 120:871-878, 1978), and that mast cell degranulation causes constriction in some vascular beds resulting in C5a-induced myocardial ischemia (Ito et al., Am. J. Physiol. 264 (Heart Circ. Physiol. 33):H1346-H1354, 1993), mast cell-dependent inflammation (Raud, J., Acta. Physiol. Scand. 135 (Suppl. 578):1-58, 1989), brain arteriole diameter constriction (Rosenblum, W. I., Brain Res. 49:75-82, 1973), and the release of allergic mediators (Ramkumar, et al., J. Biol. Chem. 268:16887-16890, 1993). Accordingly, if a method were invented to specifically inhibit mast cell degranulation, a treatment or preventative method would be demonstrated for all of the above known and many yet to be defined disease states associated with adenosine induced mast cell degranulation.

We therefore tested the hypothesis that mast cells on the abluminal surface of the arteriole serve as foci of the constrictor response. We first tested this hypothesis by directly stimulating degranulation. Application of 2 $\mu\text{g/mL}$ of compound 48/80, a mast cell secretagogue, induced a constriction similar to that observed with adenosine. Pretreatment of vessels with 10 μM cromolyn (sodium cromoglycate, a mast cell stabilizer) blocked the constrictor response induced by compound 48/80, see figures 18 and 19. Inhibition of degranulation was confirmed by observation of intact methylene blue-stained mast cells.

Adenosine A3 receptors have recently been found on mast cells. We tested the involvement of A3 receptors in the following manner: Treatment of the arterioles with the agonist IABA (30 μM , applied via pipette) resulted in vasoconstriction (see figure 17). Pretreatment with 10 μM BWA1433, characterized herein as an A3

- 58 -

specific adenosine receptor antagonist, resulted in complete abolition of the constrictor response and inhibition of mast cell degranulation (see figure 20). The A1 specific antagonist, 8PST (10 μ M) had no effect on the IABA induced vasoconstriction, proving that the constrictor
5 response is A3 receptor mediated and that this response can be blocked by contacting the A3 receptors exhibited on the mast cells with an A3 specific antagonist.

We therefore have shown that specific blockade of the A3 subtype of the adenosine receptor is effective to block the
10 vasoconstrictive response induced through adenosine activation of this receptor subtype. We have also shown that use of a specific inhibitor of the A3 adenosine receptor subtype is effective to inhibit effects induced by adenosine mediated mast cell degranulation, and have therefore shown that disease states including but not limited to myocardial
15 ischemia (Ito et al., Am. J. Physiol. 264 (Heart Circ. Physiol. 33):H1346-H1354, 1993), mast cell-dependent inflammation (Raud, J., Acta. Physiol. Scand. 135 (Suppl. 578):1-58, 1989), brain arteriole diameter constriction (Rosenblum, W. I., Brain Res. 49:75-82, 1973), and the release of allergic mediators (Ramkumar, et al., J. Biol. Chem.
20 268:16887-16890, 1993), are all amenable to prevention and treatment by contacting A3 receptor bearing mast cells with an amount of a selective A3 inhibitor effective to prevent mast cell degranulation.

EXAMPLE 15

25 EFFECT OF IABOPX ON MYOCARDIAL INFARCT SIZE IN ANESTHETIZED DOGS

The effect of 3-(3-iodo-4-aminobenzyl)-8-oxyacetate-1-propyl-xanthine (IABOPX), a xanthine adenosine receptor antagonist with high affinity for canine A3 adenosine receptors ($K_i=5.49$ nM), on
30 myocardial infarct size was compared to a vehicle-treated control group in barbitol-anesthetized dogs subjected to 90 minutes of left anterior descending (LAD) coronary artery occlusion followed by 3 hours of reperfusion. Vehicle (5 μ M NaOH in isotonic saline) or IABOPX (2 μ M) were infused at a rate of 1 ml/min directly into the LAD coronary

- 59 -

artery distal to the occlusion site beginning 10 minutes before occlusion and were continued throughout the entire ischemic period. The myocardial region at risk and infarct size were determined by the triphenyltetrazolium histochemical technique and regional myocardial blood flow by radioactive microspheres. Coronary sinus LDH activity and histamine concentrations were measured at various times throughout the experiments and myeloperoxidase activity (MPO) was determined at the conclusion of the experiments as an index of neutrophil infiltration into the ischemic-reperfused myocardium. Ten dogs were included in this preliminary study, 4 in the vehicle-treated group and 6 in the IABOPX-treated group.

In all animals, arrhythmias were encountered during occlusion and reperfusion and 3 of 4 dogs progressed to ventricular fibrillation requiring cardioversion in the vehicle-treated group and 3 of 6 in the IABOPX-treated group (Table 1). Cardioversion was unsuccessful in 2 experiments in which the drug was administered thus data analysis was performed in 8 animals, 4 in each group. Hemodynamics (Table 2) and regional myocardial blood flow in the non-ischemic left circumflex coronary artery region (Table 3) were not different between groups at baseline or during occlusion although during reperfusion dP/dt was significantly improved and LAD coronary artery blood flow was significantly decreased in IABOPX-treated dogs. In contrast, in the ischemic-reperfused region collateral blood flow during the occlusion period, the major determinant of ultimate infarct size, was slightly greater in drug-treated dogs particularly in the subepicardial region (Table 4, Figure 1). The area at risk of infarction was not different between groups: vehicle, $33.3 \pm 5.0\%$; IABOPX, $26.8 \pm 2.4\%$. however, myocardial infarct size expressed as a percent of the area at risk (vehicle, 48.1 ± 2.5 ; 20.4 ± 11.2) was significantly smaller in the IABOPX-treated group (Figure 2) and this beneficial effect of IABOPX did not appear to be the result of enhanced collateral blood flow (Figure 3). In addition, coronary sinus LDH activity (Figure 4) and histamine concentrations (Figure 5) were strikingly reduced in the

- 60 -

IABOPX-treated group during reperfusion. Finally, MPO activity was not different between the two groups although there was a tendency for reduced activity in drug-treated animals in infarcted tissue (Figure 6).

Taken together, these preliminary results suggest that IABOPX effectively reduces myocardial infarct size in anesthetized dogs by a direct cardioprotective action most likely by inhibiting mast cell degranulation secondary to blockade of A₃ adenosine receptors.

TABLE 1
Mortality and Incidence of Ventricular Fibrillation

	MORTALITY	FIBRILLATIONS		
		Occ	Rep	Total
VEHICLE	0/4	3/4	1/4	3/4
IABOPX	2/6	1/6	2/6	3/6

- 61 -

TABLE 2
HEMODYNAMICS

		HR (beat/min)	MAP (mm Hg)	LVd/dt (mm Hg/sec)	CBF (ml/min)
5	BASELINE				
	VEHICLE	134 ± 10	103 ± 6	488 ± 16	35 ± 4
	IABOPX	124 ± 11	96 ± 7	544 ± 62	33 ± 5
	OCCLUSION (75 min)				
10	VEHICLE	130 ± 11	113 ± 7	417 ± 14	-----
	IABOPX	121 ± 9	99 ± 5	464 ± 53	-----
	REPERFUSION (5 min)				
	VEHICLE	129 ± 8	96 ± 6	384 ± 45	101 ± 14 [†]
	IABOPX	128 ± 8	96 ± 5	448 ± 49 [†]	99 ± 8 [†]
15	REPERFUSION (3 hr)				
	VEHICLE	114 ± 9 [†]	99 ± 5	346 ± 19 [†]	45 ± 4
	IABOPX	116 ± 13	108 ± 4 [†]	494 ± 54 [*]	31 ± 2 [*]

All values are the mean ± SEM (Vehicle n=4; IABOPX n=4)

HR=heart rate; MAP=mean arterial blood pressure;

LVdP/dt=left ventricular dP/dt; CBF=LAD coronary blood flow

^{*}p<0.05 vs. vehicle group (ANOVA and Fisher's LSD)

[†]p<0.05 vs. baseline value within groups (ANOVA with repeated measures and Dunnet's t-test)

TABLE 3
REGIONAL MYOCARDIAL BLOOD FLOW
IN THE NORMAL REGION

		MYOCARDIAL BLOOD FLOW (ml/min/g)		
		EPI	MID	ENDO
30	BASELINE			
	VEHICLE	1.04 ± 0.04	0.96 ± 0.02	1.01 ± 0.04
	IABOPX	0.96 ± 0.08	0.91 ± 0.04	0.94 ± 0.04

- 62 -

OCCLUSION (75 min)				
	VEHICLE	0.98 ± 0.04	0.92 ± 0.09	0.97 ± 0.03
	IABOPX	1.05 ± 0.09	0.93 ± 0.05	1.30 ± 0.20
REPERFUSION (3 hr)				
5	VEHICLE	1.04 ± 0.03	0.96 ± 0.04	0.95 ± 0.06
	IABOPX	1.16 ± 0.15	1.15 ± 0.18	1.17 ± 0.18

All values are the mean ± S.E.M. (Vehicle n=4; IABOPX n=4)
 EPI=epicardium; MID=midmyocardium; ENDO=endocardium

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TABLE 4
 REGIONAL MYOCARDIAL BLOOD FLOW
 IN THE ISCHEMIC REGION

MYOCARDIAL BLOOD FLOW				
(ml/min/g)				
		EPI	MID	ENDO
BASELINE				
	VEHICLE	0.97 ± 0.04	0.84 ± 0.04	0.85 ± 0.02
	IABOPX	0.95 ± 0.13	0.76 ± 0.06	0.71 ± 0.05*
20	OCCLUSION (75 min)			
	VEHICLE	0.20 ± 0.04 [†]	0.11 ± 0.04 [†]	0.08 ± 0.04 [†]
	IABOPX	0.47 ± 0.10* [†]	0.22 ± 0.06 [†]	0.13 ± 0.04 [†]
REPERFUSION (3 hr)				
	VEHICLE	0.95 ± 0.14	1.29 ± 0.17 [†]	2.00 ± 0.28 [†]
25	IABOPX	1.11 ± 0.14	0.86 ± 0.07	1.43 ± 0.21 [†]

All values are the mean ± S.E.M. (Vehicle n=4; IABOPX n=4)

EPI=epicardium; MID=midmyocardium; ENDO=endocardium

*p<0.05 vs. vehicle group (ANOVA and Fisher's LSD)

30 [†]p<0.05 vs. baseline value within groups (ANOVA with repeated measures and Dunnet's t-test)

While the foregoing specification teaches the principles of the present invention, with examples provided for the purpose of illustration, it will be understood that the practice of the invention

- 63 -

encompasses all of the usual variations, adaptations, modifications, as come within the scope of the following claims and its equivalents.

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- 64 -

WHAT IS CLAIMED IS:

1. A method for achieving blockade of the vasoconstrictive response induced through adenosine activation of the A3 adenosine receptor subtype which comprises contacting cells bearing said receptor with an amount of an adenosine A3 receptor subtype specific inhibitor effective to block activation of said receptor by adenosine.
2. A method for treating or preventing myocardial ischemia, inflammation, brain arteriole diameter constriction, and the release of allergic mediators, which comprises using a specific inhibitor of the A3 adenosine receptor subtype to inhibit effects induced by adenosine mediated mast cell degranulation by contacting A3 receptor bearing mast cells with an amount of a selective A3 inhibitor effective to prevent mast cell degranulation.
3. A method for preventing or treating asthma, bronchoconstriction, allergic potentiation, inflammation or reperfusion injury in a human which comprises administering an amount of a xanthine or a xanthine derivative having an acidic aryl at the 8 position and a substituted or unsubstituted aryl, alkyl or alkenyl substituent at the 3 position effective to antagonize activation of the adenosine receptor of the A3 subtype by adenosine.
4. A method for preventing mast cell degranulation in a human which comprises administering an amount of a xanthine or a xanthine derivative having an acidic aryl at the 8 position and a substituted or unsubstituted aryl, alkyl or alkenyl substituent at the 3 position effective to antagonize activation of the adenosine receptor of the A3 subtype by adenosine.
5. A method for achieving blockade of vascular constriction induced through activation of the A3 subtype of the

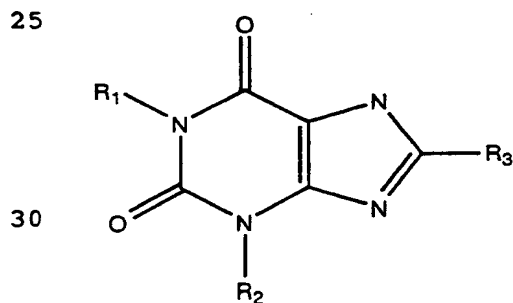
- 65 -

adenosine receptor in a primate which comprises contacting said
receptor in the vasculature with an amount of a xanthine or xanthine
derivative effective to reduce vasoconstriction in the vasculature without
any substantial effect (binding or blockade) of the A1 or A2 subtypes of
5 the adenosine receptor.

6. The method of claim 5 which comprises the
treatment or prevention of disease states mediated through activation of
the A3 subtype of the adenosine receptor on mast cells by prevention of
10 mast cell degranulation through blockade of the A3 subtype of the
adenosine receptor by contacting mast cells with an inhibitory effective
amount of a xanthine or xanthine derivative specific for the A3 receptor
subtype.

7. The method of claim 6 wherein the disease state
associated with A3 adenosine receptor activation and mast cell
degranulation includes asthma, myocardial reperfusion injury, allergic
reactions including but not limited to rhinitis, poison ivy induced
responses, urticaria, scleroderma, arthritis, and inflammatory bowel
20 diseases.

8. The method of any one of Claims 3-7 wherein said
xanthine has the formula:



- 66 -

wherein R₁, R₂, and R₃, independently, are as defined below:

<u>R₁</u>	<u>R₂</u>	<u>R₃</u>
alkyl	aryl	acidic aryl
alkenyl	substituted aryl	substituted acidic aryl
cycloalkyl	hetero-aryl	
	substituted hetero-aryl	

wherein:

alkyl, alkenyl, cycloalkyl is substituted or unsubstituted

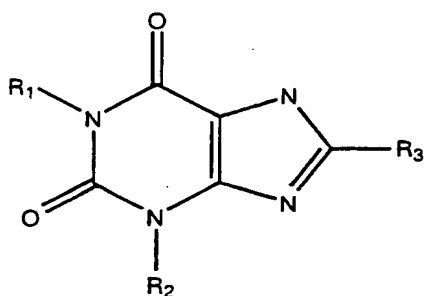
aryl is benzyl, phenyl;

substituted aryl is an aryl substituted with an alkyl, amino or halogen;

and

acidic aryl is an aryl substituted with a carboxylate, oxyacetate, acrylate, sulphonate, phosphonate, or tetrazol.

9. The method of claim 8 wherein said xanthine is:

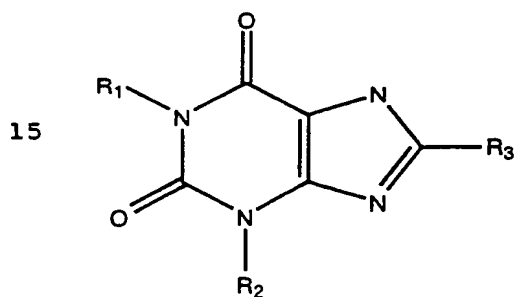


- 67 -

wherein R₁, R₂, and R₃, independently, are as defined below:

	<u>R₁</u>	<u>R₂</u>	<u>R₃</u>
5	lower alkyl	benzyl halogenated benzyl amino-benzyl halogenated amino-benzyl.	benzyl-acid

10. The method of Claim 9 wherein said xanthine is:



wherein R₁, R₂, and R₃, independently, are as defined below:

	<u>R₁</u>	<u>R₂</u>	<u>R₃</u>
25	-C ₃ H ₇ -CH ₃ -C ₂ H ₅	-C ₃ H ₇ -benzyl -halogenated benzyl -aminobenzyl -halogenated aminobenzyl	-CH ₂ -C ₆ H ₄ -O-acid -CH ₂ -COO ⁻ -indole
30			

wherein said acid is -indole, -carboxylate, sulphonate, phosphonate.

- 68 -

11. The method of any one of claims 3-5 wherein the xanthine is selected from the group consisting of LABOPX, BW-A1433, BW-934, and BW-A215.

5 12. A method for preventing or treating asthma, bronchoconstriction, allergic potentiation, inflammation or reperfusion injury in a human which comprises administering an amount of a xanthine or a xanthine derivative having an affinity for the A3 subtype
10 of the human adenosine receptor which is at least one order of magnitude greater than the affinity for either the A1 A2a or A2b subtypes of the human adenosine receptor effective to antagonize activation of the adenosine receptor of the A3 subtype by adenosine.

15 13. A method for preventing mast cell degranulation in a human which comprises administering an amount of a xanthine or a xanthine derivative having an affinity for the A3 subtype of the human adenosine receptor which is at least one order of magnitude greater than
20 the affinity for either the A1 or A2 subtypes of the human adenosine receptor effective to antagonize activation of the adenosine receptor of the A3 subtype by adenosine.

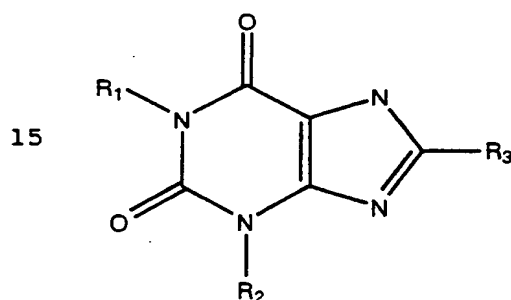
 14. A method for achieving blockade of vascular constriction induced through activation of the A3 subtype of the adenosine receptor in a primate which comprises contacting said
25 receptor in the vasculature with an amount of a xanthine or xanthine derivative having an affinity for the A3 subtype of the human adenosine receptor which is at least one order of magnitude greater than the affinity for either the A1 A2a or A2b subtypes of the human adenosine
30 receptor effective to reduce vasoconstriction in the vasculature without any substantial effect (binding or blockade) of the A1 A2a or A2b subtypes of the adenosine receptor.

 15. The method of claim 14 wherein said vasculature is in the lung.

- 69 -

16. A method for achieving blockade of the A3 subtype of the adenosine receptor in a primate which comprises contacting said receptor with an amount of a xanthine or xanthine derivative effective to inhibit activation of said receptor by adenosine, without any substantial effect (binding or blockade) of other subtypes of the adenosine receptor.

17. The method of Claim 16 wherein said xanthine has the formula:



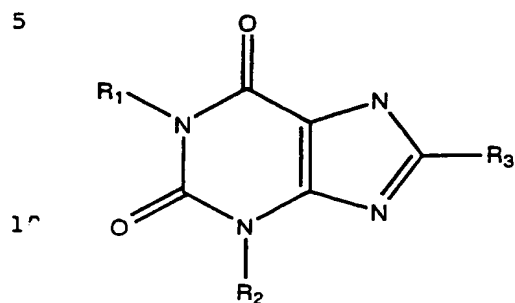
wherein R₁, R₂, and R₃, independently, are as defined below:

<u>R₁</u>	<u>R₂</u>	<u>R₃</u>
alkyl	aryl	acidic aryl
alkenyl	substituted aryl	substituted acidic aryl
cycloalkyl	hetero-aryl	
	substituted hetero-aryl	

wherein:
 alkyl, alkenyl, cycloalkyl is substituted or unsubstituted
 aryl is benzyl, phenyl;
 substituted aryl is an aryl substituted with an alkyl, amino or halogen;
 and
 acidic aryl is an aryl substituted with a carboxylate, oxyacetate, acrylate, sulphate, phosphonate, or tetrazol.

- 70 -

18. The method of claim 16 wherein said xanthine is:



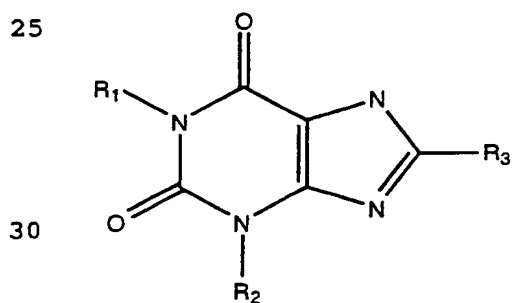
wherein R₁, R₂, and R₃, independently, are as defined below:

15

<u>R₁</u>	<u>R₂</u>	<u>R₃</u>
lower alkyl	benzyl halogenated benzyl amino-benzyl halogenated amino-benzyl.	benzyl-acid

20

19. The method of Claim 16 wherein said xanthine is:



- 71 -

wherein R₁, R₂, and R₃, independently, are as defined below:

	<u>R₁</u>	<u>R₂</u>	<u>R₃</u>
5	-C ₃ H ₇	-C ₃ H ₇	-CH ₂ -C ₆ H ₄ -O-acid
	-CH ₃	-benzyl	-CH ₂ -COO ⁻
	-C ₂ H ₅	-halogenated	-indole
		benzyl	
		-aminobenzyl	
10		-halogenated	
		aminobenzyl	

wherein said acid is -indole, -carboxylate, sulphonate, phosphonate.

20. The method of Claim 16 wherein the xanthine is
15 selected from the group consisting of LABOPX, BW-A1433, BW-934,
and BW-A215.

21. The method of Claim 16 wherein the xanthine has a
20 pK_i for the A₃ subtype of 7 or greater, and a pK_i for other adenosine
receptor subtypes of 6 or less.

22. A method for achieving blockade of the A₃ subtype
of the adenosine receptor in a primate which comprises contacting said
25 receptor with an amount of an A₃ adenosine receptor antagonist having
a pK_i for the A₃ subtype of 7 or greater, and a pK_i for other adenosine
receptor subtypes of 6 or less.

23. A method for treating an autoimmune disease
30 selected from the group consisting of Addison's disease (adrenal),
autoimmune hemolytic anemia (red cells), Crohn's disease (gut),
Goodpasture's syndrome (kidney and lungs), Grave's disease (thyroid),
Hashimoto's thyroiditis (thyroid), idiopathic thrombocytopenic purpura
(platelets), Insulin-dependent diabetes militus (pancreatic beta cells),
multiple sclerosis (brain and spinal cord), myasthenia gravis

- 72 -

(nerve/muscle synapses), Pemphigus vulgaris (skin), pernicious anemia (gastric parietal cells), poststreptococcal glomerulonephritis (kidney), psoriasis (skin), rheumatoid arthritis (connective tissue), scleroderma (heart, lung, gut, kidney), Sjogren's syndrome (liver, kidney, brain, thyroid, salivary gland), spontaneous infertility (sperm), and systemic lupus erythematosus (DNA, platelets, other tissues), which comprises administration of selective A3 adenosine receptor antagonists effective to inhibit mast cell degranulation.

10

15

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25

30

FIG. 1

2/31

10	atgccgccct	30	gcctacatcg	50	gctcatcgcc
70	tgcccgga	90	atctggcg	110	ccaggcgctg
130	cgggatgcca	150	ctggcggtgg	170	cgtgggtgcc
190	ctgggtcatcc	210	agacctactt	230	ccacacctgc
250	ctcatggttg	270	ccatcctggc	290	cctgctggca
310	attgctgtgg	330	ggtaacaagt	350	ggtggtgacc
370	ccccggagg	390	tctccttcgt	410	ggtgggactg
430	accctatgt	450	gggcctgggc	470	agccaacggc
490	agcatggggg	510	tcatacagcat	530	ggagtacatg
550	gtctacttca	570	tcctcatggt	590	cctcatctac
610	ctggagggtct	630	aggtgtcggc	650	ctcctccggc
670		690		710	

FIG. 2A

3/31

gacccgcaga agtactatgg gaaggagctg aagatcgcca agtcgctggc cctcatcctc
730 750 770
ttcctctttg ccttcagctg gctgcctttg cacatcctca actgcacac cctcttctgc
790 810 830
ccgtcctgcc acaagcccag catcctacc tacattgcca tcttcctcac gcacggcaac
850 870 890
tcggccatga accccattgt ctatgccttc cgcaccaga agttccgct cacttcctt
910 930 950
aagatttgga atgaccattt ccgctgccag cctgcacctc ccattgacga ggatctccca
970
gaagagaggg ctgatgacta g

FIG. 2B

4 / 31

10 20
 Met Pro Ile Met Gly Ser Ser Val Tyr Ile Thr Val Glu Leu Ala Ile Ala Val Leu Ala
 30 40
 Ile Leu Gly Asn Val Leu Val Cys Trp Ala Val Trp Leu Asn Ser Asn Leu Gln Asn Val
 50 60
 Thr Asn Tyr Phe Val Val Ser Leu Ala Ala Asp Ile Ala Val Gly Val Leu Ala Ile
 70 80
 Pro Phe Ala Ile Thr Ile Ser Thr Gly Phe Cys Ala Ala Cys His Gly Cys Leu Phe Ile
 90 100
 Ala Cys Phe Val Leu Val Leu Thr Gln Ser Ser Ile Phe Ser Leu Leu Ala Ile Ala Ile
 110 120
 Asp Arg Tyr Ile Ala Ile Arg Ile Pro Leu Arg Tyr Asn Gly Leu Val Thr Gly Thr Arg
 130 140
 Ala Lys Gly Ile Ile Ala Ile Cys Trp Val Leu Ser Phe Ala Ile Gly Leu Thr Pro Met
 150 160
 Leu Gly Trp Asn Asn Cys Gly Gln Pro Lys Glu Gly Lys Asn His Ser Gln Gly Cys Gly
 170 180
 Glu Gly Gln Val Ala Cys Leu Phe Glu Asp Val Val Pro Met Asn Tyr Met Val Tyr Phe
 190 200
 Asn Phe Phe Ala Cys Val Leu Val Pro Leu Leu Met Leu Gly Val Tyr Leu Arg Ile
 210 220
 Phe Leu Ala Ala Arg Arg Gln Leu Lys Gln Met Glu Ser Gln Pro Leu Pro Gly Glu Arg
 230 240
 Ala Arg Ser Thr Leu Gln Lys Glu Val His Ala Ala Lys Ser Leu Ala Ile Ile Val Gly
 250 260
 Leu Phe Ala Leu Cys Trp Leu Pro Leu His Ile Ile Asn Cys Phe Thr Phe Phe Cys Pro
 270 280
 Asp Cys Ser His Ala Pro Leu Trp Leu Met Tyr Leu Ala Ile Val Leu Ser His Thr Asn
 290 300
 Ser Val Val Asn Pro Phe Ile Tyr Ala Tyr Arg Ile Arg Glu Phe Arg Gln Thr Phe Arg
 310 320
 Lys Ile Ile Arg Ser His Val Leu Arg Gln Gln Glu Pro Phe Lys Ala Ala Gly Thr Ser
 330 340
 Ala Arg Val Leu Ala Ala His Gly Ser Asp Gly Glu Gln Val Ser Leu Arg Leu Asn Gly
 350 360
 His Pro Pro Gly Val Trp Ala Asn Gly Ser Ala Pro His Pro Glu Arg Arg Pro Asn Gly
 370 380
 Tyr Ala Leu Gly Leu Val Ser Gly Gly Ser Ala Gln Glu Ser Gln Gly Asn Thr Gly Leu
 390 400
 Pro Asp Val Glu Leu Leu Ser His Glu Leu Lys Gly Val Cys Pro Glu Pro Pro Gly Leu
 410
 Asp Asp Pro Leu Ala Gln Asp Gly Ala Gly Val Ser

FIG. 3

SUBSTITUTE SHEET (RULE 26)

5/31

10	atgccatca	10	tggtgtacatc	30	acggtggagc	50	tgtgctggcc
70	atcctgggca	90	gtgctgggcc	110	acagcaacct	130	gcagaacgctc
130	acaaactact	150	actggcgccg	170	gccgacatcg	190	gctcgccatc
190	ccctttgcca	210	tcacctcag	230	tgcgctgcct	250	cctcttcatt
250	gcctgcttcg	270	tcctggctct	290	gccacggctg	310	catcgccatt
310	gaccgctaca	330	cacgcagagc	350	gtctcctggc	370	cggcacgagg
370	gctaagggca	390	catcccgctc	410	gcttggtgac	430	gactccccatg
430	ctaggttgga	450	ctgctgggtg	470	ccatcggcct	490	gggctgcggg
490	gagggccaaag	510	tcaggccaaag	530	accactccca	550	ggtgtacttc
550	aacttctttg	570	ctttgaggat	590	tgaactacat	610	tttgcggtatc
610	ttcctggcgg	630	ggtgccccctg	650	tggtgtgtcta	670	gggggagcgg
670	gctgaagcag	690	atggagagcc	710	agcctctgccc		

FIG. 4A

6/31

```

gcacgggtcca cactgcagaa ggaggtccat gctgccaagt cactggccat cattgtggg
730          750
ctctttgccc tctgtgtgct gcccctacac atcatcaact gcttcacttt cttctgcccc
790          810
gactgcagcc acgcccctct ctggctcatg tacctggcca tcgtcctctc ccacaccaat
850          870
tcggttgtga atcccttcat ctacgcctac cgtatccgcg agttccgccca gaccttccgc
910          930
aagatcattc gcagccacgt cctgaggcag caagaacctt tcaaggcagc tggcaccagt
970          990
gcccggtctt tggcagctca tggcagtgac ggagagcagg tcagcctccg tctcaacggc
1030         1050
caccgcccag gagtgtgggc caacggcagt gctccccacc ctgagcggag gcccaatggc
1090         1110
tatgccctgg ggctggtgag tggaggagat gcccaagagt ccagggggaa cacggggcctc
1150         1170
ccagacgtgg agctcccttag ccatgagctc aagggagtgt gccagagcc ccctggccta
1210         1230
gatgaccccc tggcccagga tggagcagga gtgtcctga

```

FIG. 4B

7 / 31

Met Leu Leu Glu Thr Gln Asp Ala Leu Tyr Val Ala Leu Glu Leu Val Ile Ala Ala Leu	10	20
Ser Val Ala Gly Asn Val Leu Val Cys Ala Ala Val Gly Thr Ala Asn Thr Leu Gln Thr	30	40
Pro Thr Asn Tyr Phe Leu Val Ser Leu Ala Ala Ala Asp Val Ala Val Gly Leu Phe Ala	50	60
Ile Pro Phe Ala Ile Thr Ile Ser Leu Gly Phe Cys Thr Asp Phe Tyr Gly Cys Leu Phe	70	80
Leu Ala Cys Phe Val Leu Val Leu Thr Gln Ser Ser Ile Phe Ser Leu Leu Ala Val Ala	90	100
Val Asp Arg Tyr Leu Ala Ile Cys Val Pro Leu Arg Tyr Lys Ser Leu Val Thr Gly Thr	110	120
Arg Ala Arg Gly Val Ile Ala Val Leu Trp Val Leu Ala Phe Gly Ile Gly Leu Thr Pro	130	140
Phe Leu Gly Trp Asn Ser Lys Asp Ser Ala Thr Asn Asn Cys Thr Glu Pro Trp Asp Gly	150	160
Thr Thr Asn Glu Ser Cys Cys Leu Val Lys Cys Leu Phe Glu Asn Val Val Pro Met Ser	170	180
Tyr Met Val Tyr Phe Asn Phe Phe Gly Cys Val Leu Pro Pro Leu Leu Ile Met Leu Val	190	200
Ile Tyr Ile Lys Ile Phe Leu Val Ala Cys Arg Gln Leu Gln Arg The Glu Leu Met Asp	210	220
His Ser Arg Thr Thr Leu Gln Arg Glu Ile His Ala Ala Lys Ser Leu Ala Met Ile Val	230	240
Gly Ile Phe Ala Leu Cys Trp Leu Pro Val His Ala Val Asn Cys Val Thr Leu Phe Gln	250	260
Pro Ala Gln Gly Lys Asn Lys Pro Lys Trp Ala Met Asn Met Ala Ile Leu Leu Ser His	270	280
Ala Asn Ser Val Val Asn Pro Ile Val Tyr Ala Tyr Arg Asn Arg Asp Phe Arg Tyr Thr	290	300
Phe His Lys Ile Ile Ser Arg Tyr Leu Leu Cys Gln Ala Asp Val Lys Ser Gly Asn Gly	310	320
Gln Ala Gly Val Gln Pro Ala Leu Gly Val Gly Leu	330	

FIG. 5

SUBSTITUTE SHEET (RULE 26)

8/31

10	atgctgctgg	30	gtggcgctgg	50	cgccgcgctt
70	agacacagga	90	agctggtcat	110	
	gcaacgtgct	150	cgcggtggga	170	tctgcagacg
130	acttcctggg	210	gcgccgacg	230	gctcttcgcc
190	ccatcaccat	270	ttctgactg	290	ctgcctcttc
250	tcgtgctgg	330	agctccatct	350	ggccgtggca
310	acctggccat	390	ctcaggata	410	cacggggacc
370	gggtcattgc	450	gtccttgct	470	attgactcca
430	ggaacagtaa	510	accaacaact	530	ctgggatgga
490	aaagctgctg	570	tgtctctttg	590	cccatgagc
550	atttcaattt	630	gttctgccc	650	aatgctggtg
610	agatcttcct	690	aggcagcttc	710	gctgatggac
670					

FIG. 6A

9/31

```
cactcgagga ccaccctcca gcgggagatc catgcagcca agtcactggc catgattgtg
730                                     770
gggatttttg ccctgtgctg gttacctgtg catgctgtta actgtgtcac tcttttccag
790                                     830
ccagctcagg gtaaaaaataa gcccaagtgg gcaatgaata tggccattct tctgtcacat
850                                     890
gccaatcag ttgtcaatcc cattgtctat gcttacgga accgagactt ccgctacact
910                                     950
tttcacaaaa ttatctccag gtatcttctc tgccaagcag atgtcaagag tgggaatggt
970                                     990
caggctgggg tacagcctgc tctcgggtgtg ggcctatga
```

FIG. 6B

10/31

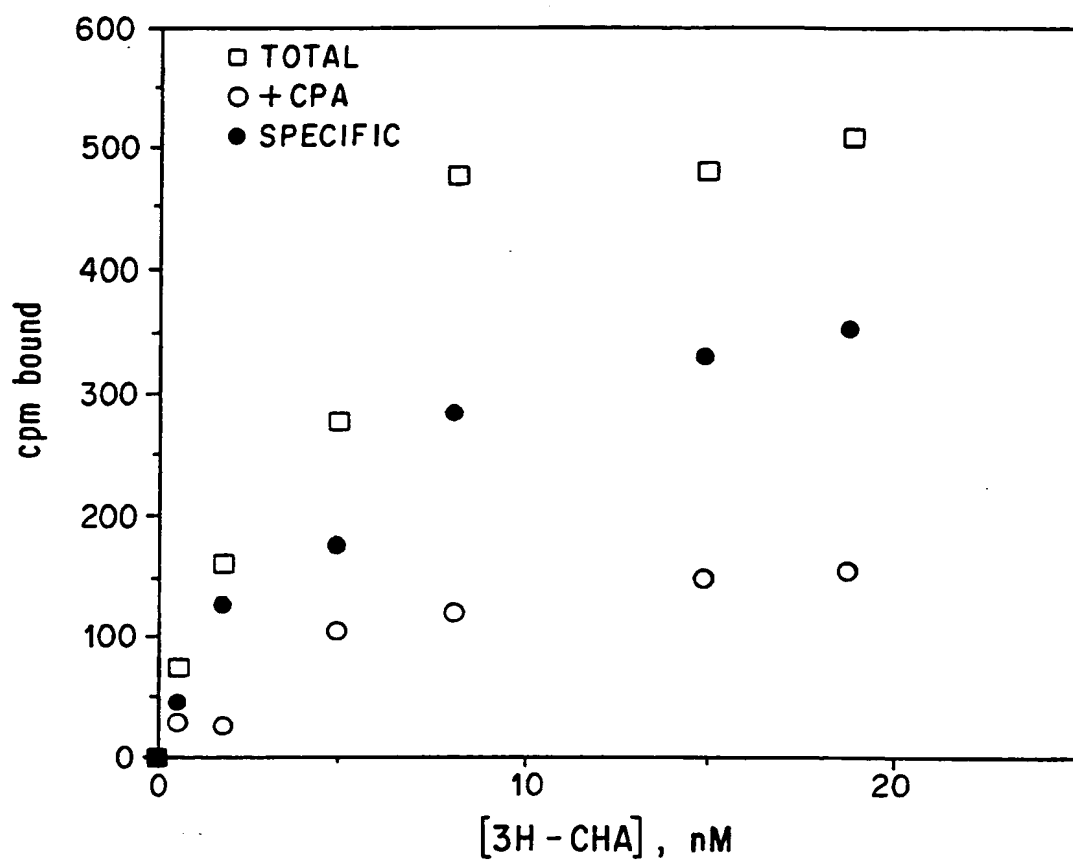


FIG. 7

11/31

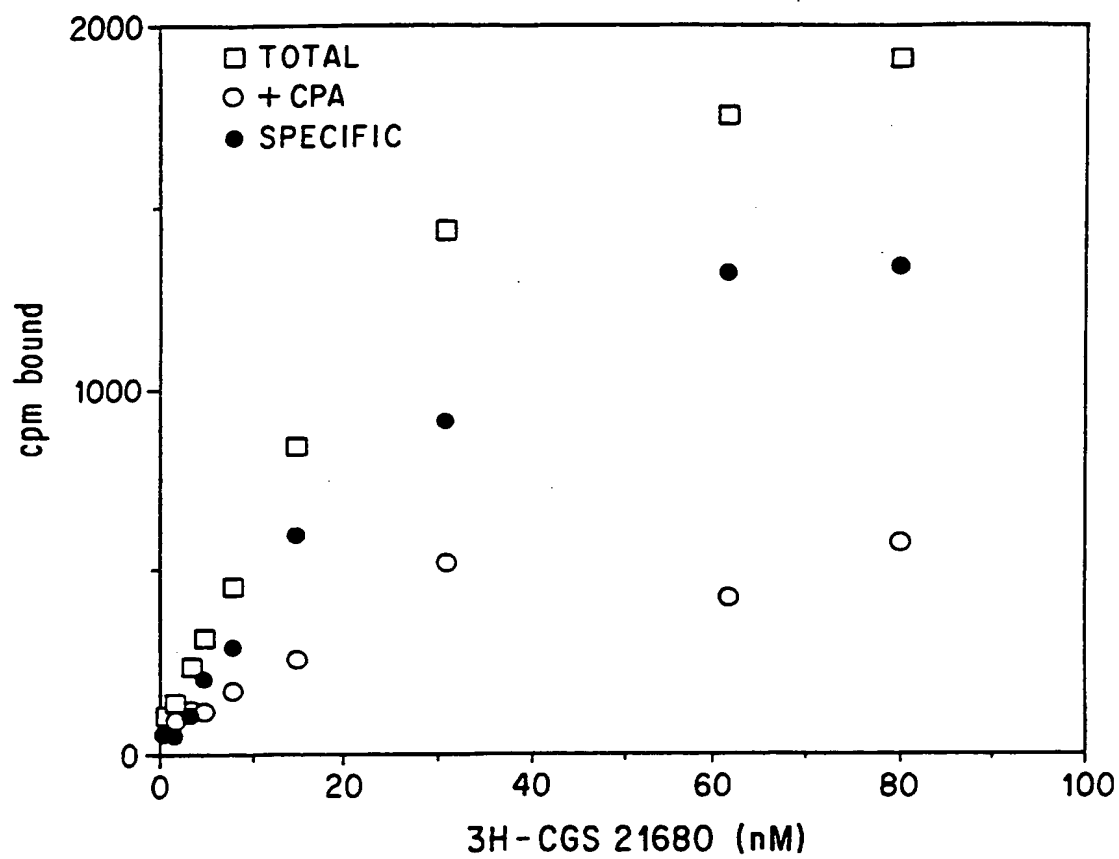


FIG. 8

12/31

Met Pro Asn Asn Ser Thr Ala Leu Ser Leu Ala Asn Val Thr Tyr Ile Thr Met Glu Ile 20
 Phe Ile Gly Leu Cys Ala Ile Val Gly Asn Val Leu Val Ile Cys Val Val Lys Leu Asn 40
 Pro Ser Leu Gln Thr Thr Phe Tyr Phe Ile Val Ser Leu Ala Leu Ala Asp Ile Ala 60
 Val Gly Val Leu Val Met Pro Leu Ala Ile Val Val Ser Leu Gly Ile Thr Ile His Phe 80
 Tyr Ser Cys Leu Phe Met Thr Cys Leu Leu Leu Ile Phe Thr His Ala Ser Ile Met Ser 100
 Leu Leu Ala Ile Ala Val Asp Arg Tyr Leu Arg Val Lys Leu Thr Val Arg Tyr Lys Arg 120
 Val Thr Thr His Arg Arg Ile Trp Leu Ala Leu Gly Leu Cys Trp Leu Val Ser Phe Leu 140
 Val Gly Leu Thr Pro Met Phe Gly Trp Asn Met Lys Leu Thr Ser Glu Tyr His Arg Asn 160
 Val Thr Phe Leu Ser Cys Gln Phe Val Ser Val Met Arg Met Asp Tyr Met Val Tyr Phe 180
 Ser Phe Leu Thr Trp Ile Phe Ile Pro Leu Val Val Met Cys Ala Ile Tyr Leu Asp Ile 200
 Phe Tyr Ile Ile Arg Asn Lys Leu Ser Leu Asn Leu Ser Asn Ser Lys Glu Thr Gly Ala 220
 Phe Tyr Gly Arg Glu Phe Lys Thr Ala Lys Ser Leu Phe Leu Val Leu Phe Leu Phe Ala 240
 Leu Ser Trp Leu Pro Leu Ser Ile Ile Asn Cys Ile Ile Tyr Phe Asn Gly Glu Val Pro 260
 Gln Leu Val Leu Tyr Met Gly Ile Leu Leu Ser His Ala Asn Ser Met Met Asn Pro Ile 280
 Val Tyr Ala Tyr Lys Ile Lys Lys Phe Lys Glu Thr Tyr Leu Leu Ile Leu Lys Ala Cys 300
 Val Val Cys His Pro Ser Asp Ser Leu Asp Thr Ser Ile Glu Lys Asn Ser Glu 310

FIG. 9

13/31

10	atgcccaaca	30	tctgtcattg	50	catggaatt
70		90		110	
130	ttcattggac	150	agtgggcaac	170	caagctgaac
190	cccagcctgc	210	cttctatttc	230	tgacattgct
250	gttgggggtgc	270	tttggccatt	290	aatccacttc
310	tacagctgcc	330	ttgcctactg	350	catcatgtcc
370	ttgctggcca	390	ccgatacttg	410	atacaagagg
430	gtcaccactc	450	atggctggcc	470	gtcattcctg
490	gtgggattga	510	tggctggaac	530	ccacagaaat
550	gtcaccttcc	570	atttgtttcc	590	ggtatacttc
610	agcttcctca	630	catccccctg	650	tcttgacatc
670	ttttacatca	690	actcagtctg	710	gacagggtgca

FIG. 10A

ttttatggac 730	gggagttcaa	gacggctaag 750	tccttgtttc	tggttctttt 770	cttgtttgct
ctgtcatggc 790	tgcctttatc	tatcatcaac 810	tgcatcatct	actttaatgg 830	tgagggtacca
cagcttggtc 850	tgtacatggg	catcctgctg 870	tcccatgcca	actccatgat 890	gaaccctatc
gtctatgcct 910	ataaaataaa	gaagttcaag 930	gaaacctacc	tttttgatcct 950	caaagcctgt
gtgggtctgcc	atccctctga	ttctttggac	acaagcattg	agaagaattc	tgagtag

14/31

FIG. 10B

15/31

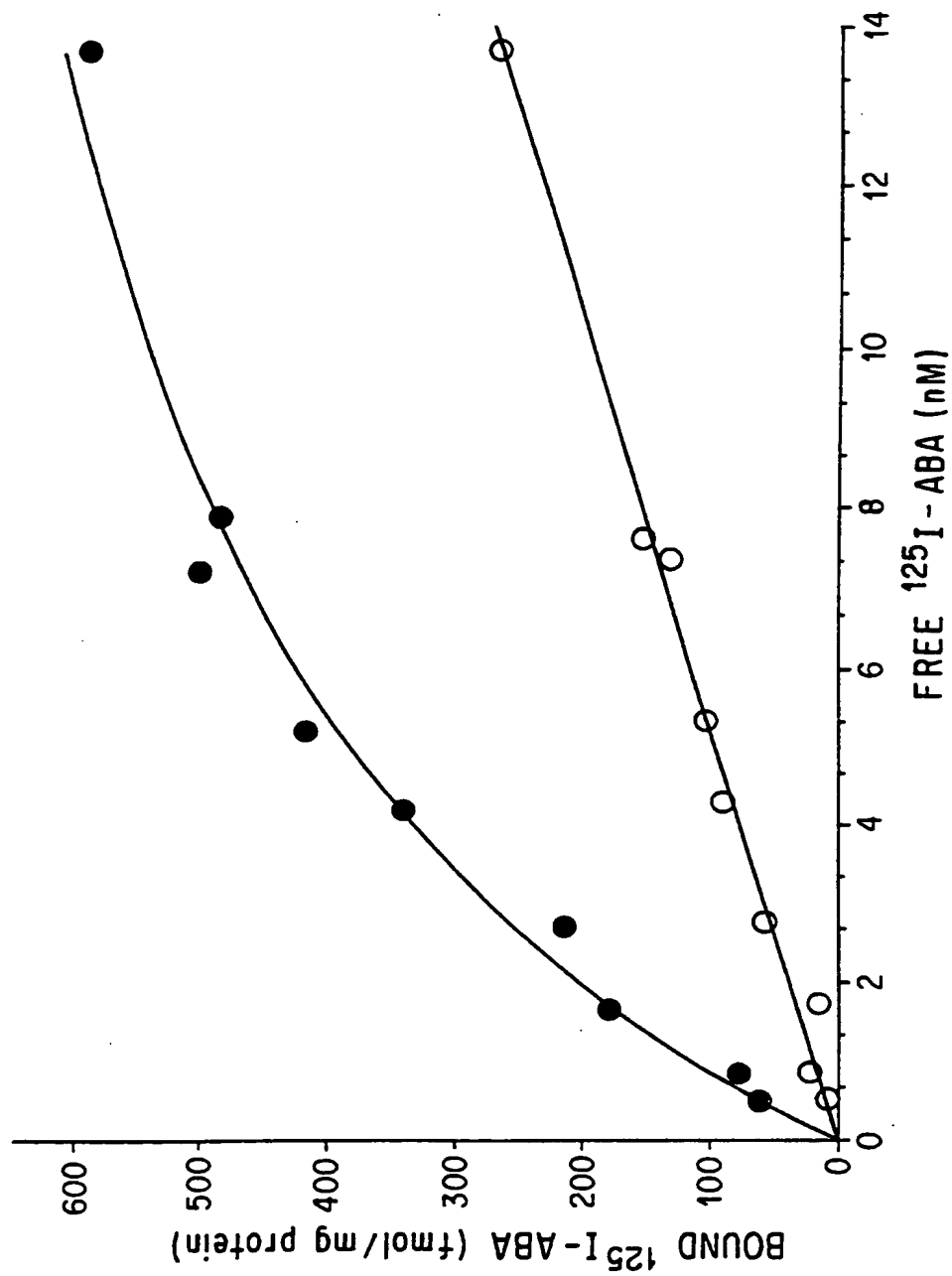


FIG. 11A

16/31

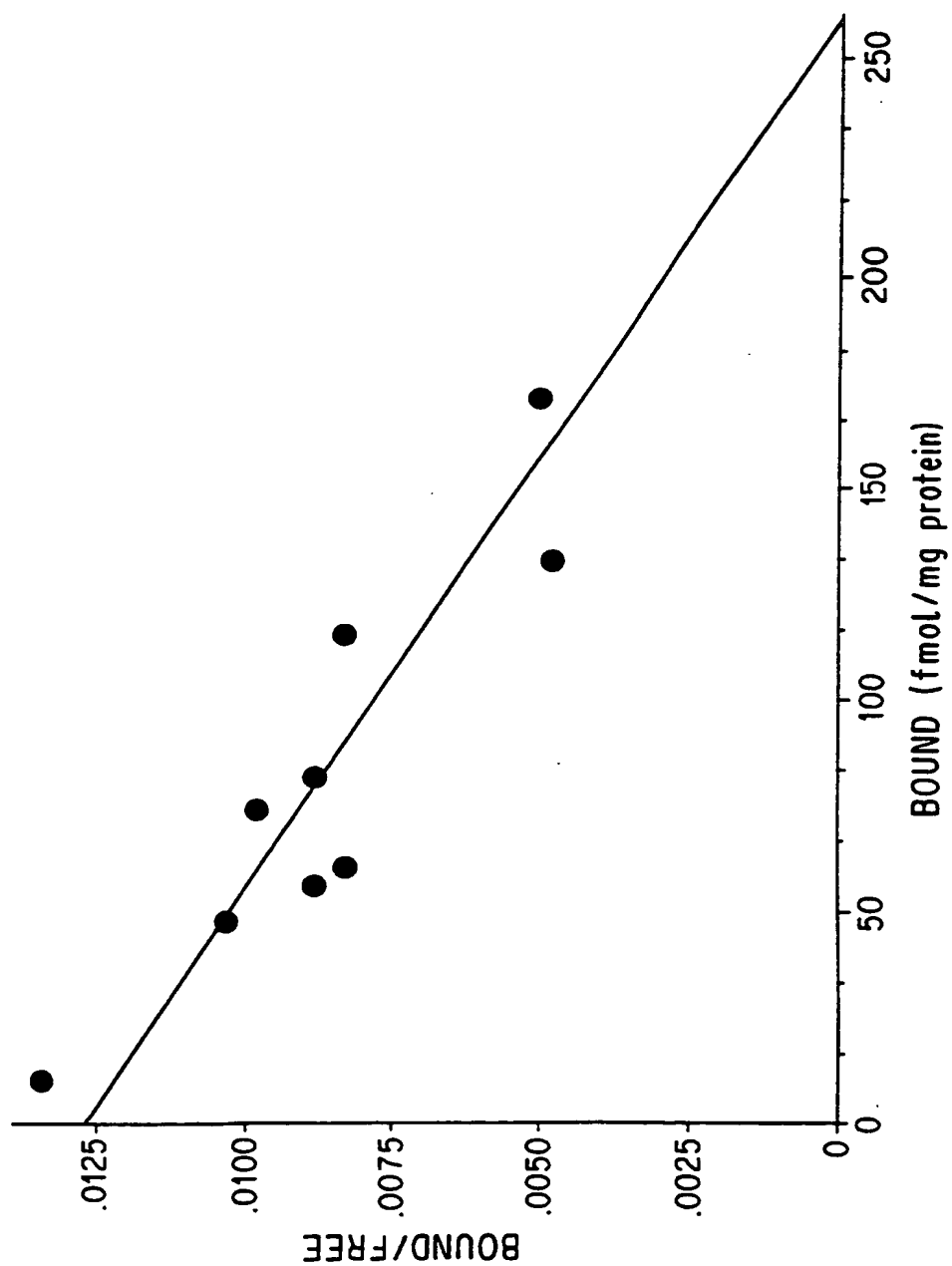


FIG. 11B

17 / 31

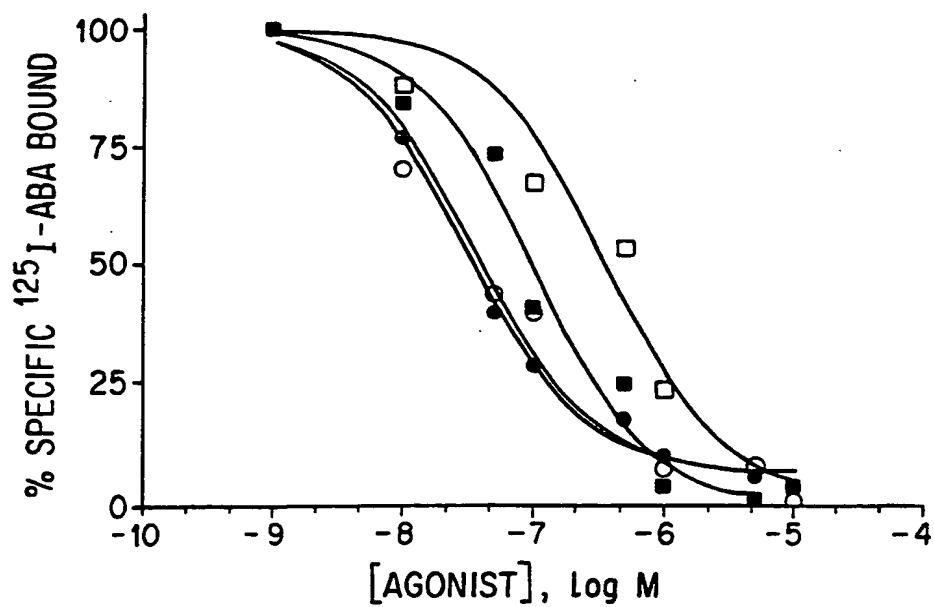


FIG. 12A

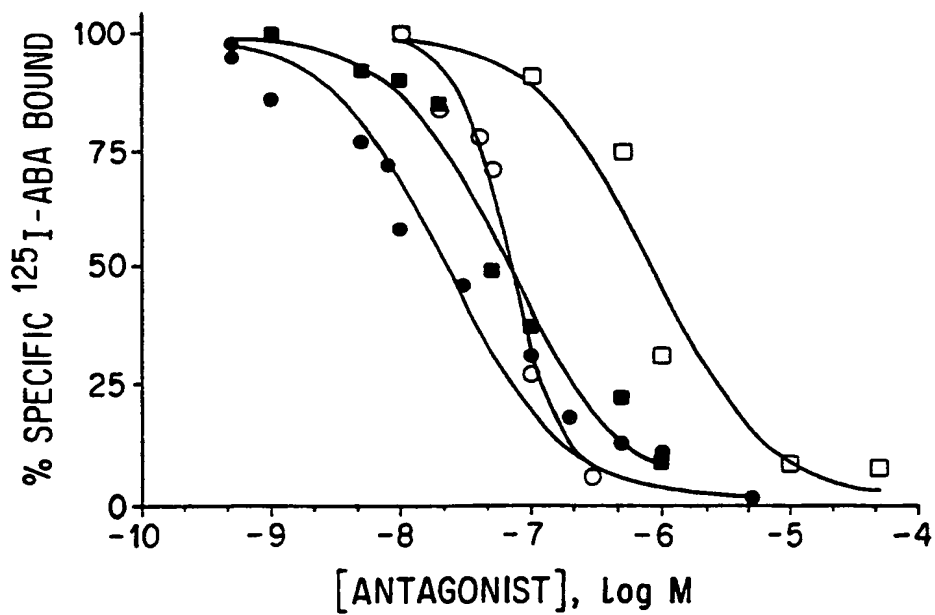


FIG. 12B

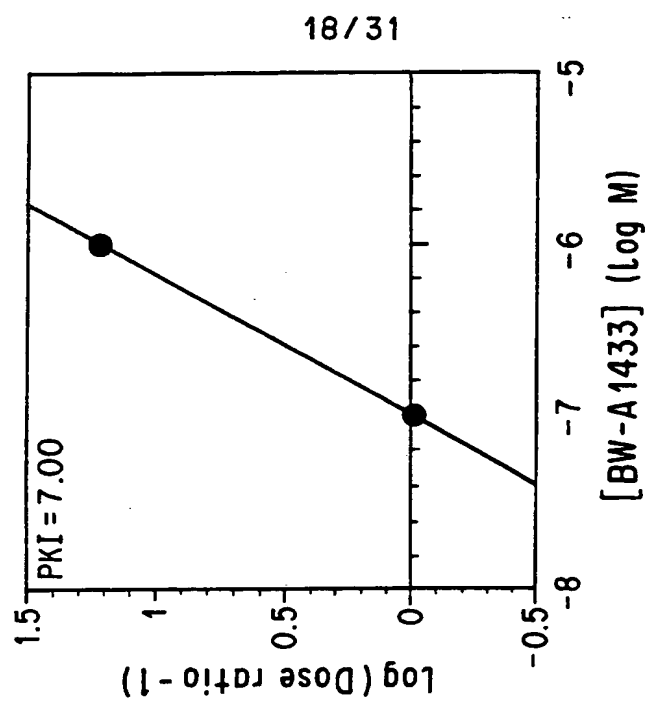


FIG. 13B

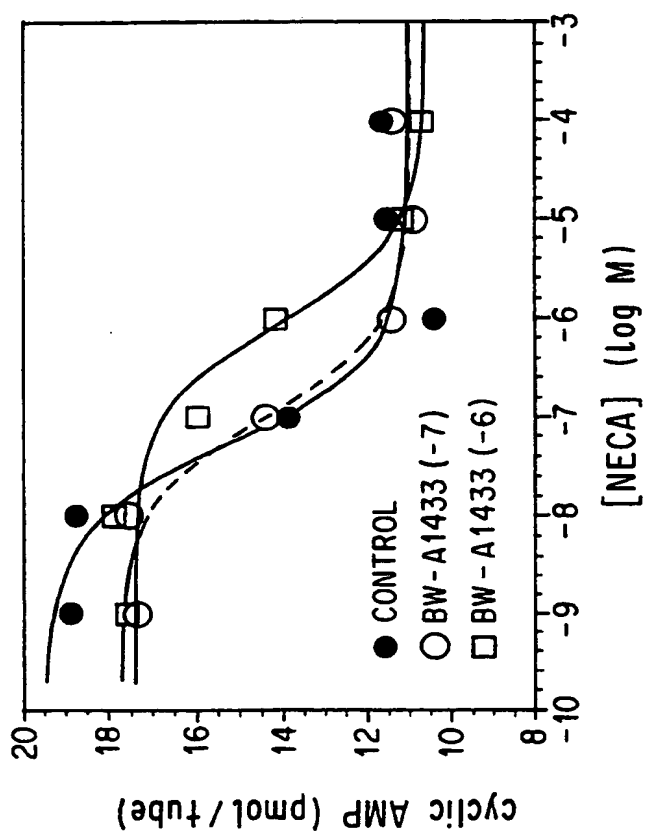


FIG. 13A

19/31

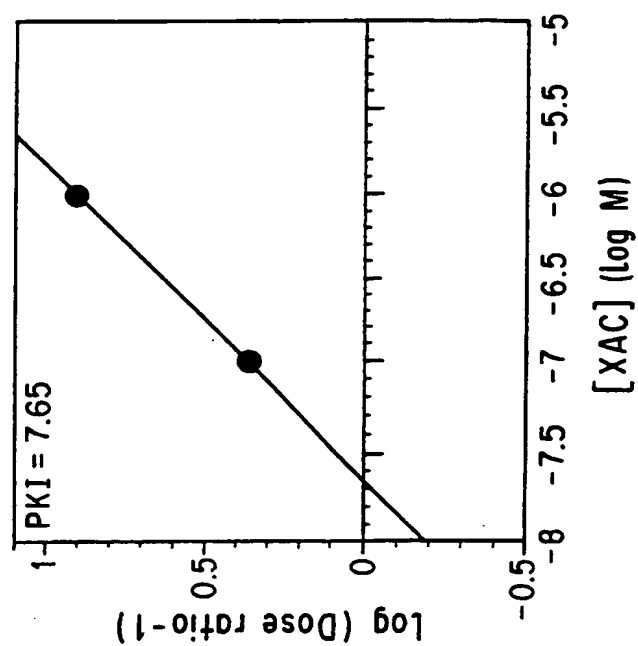


FIG. 13D

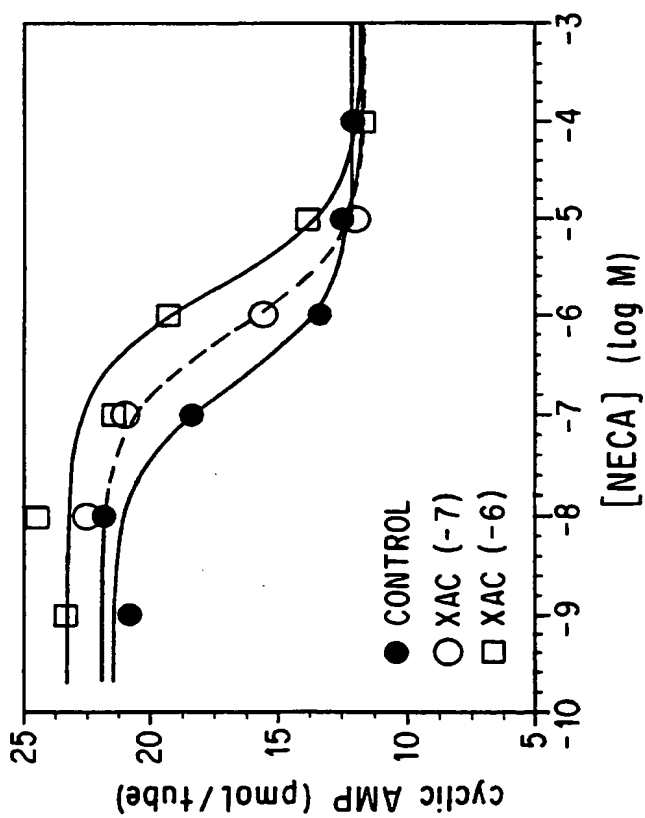


FIG. 13C

SUBSTITUTE SHEET (RULE 26)

20/31

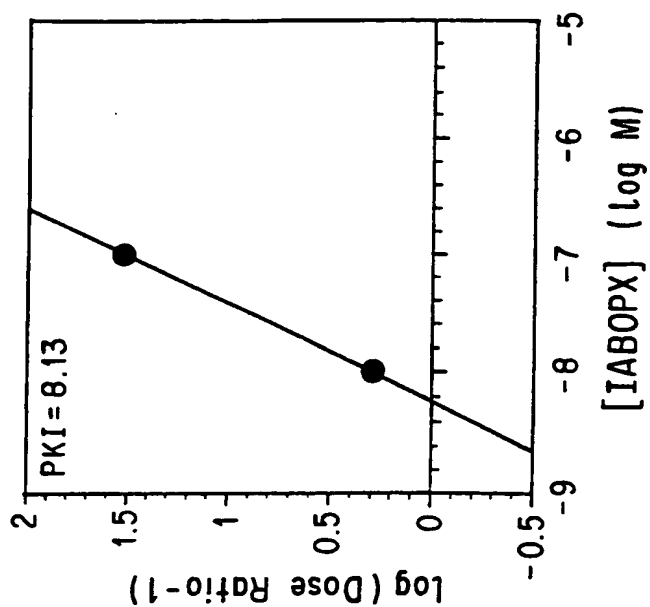


FIG. 13F

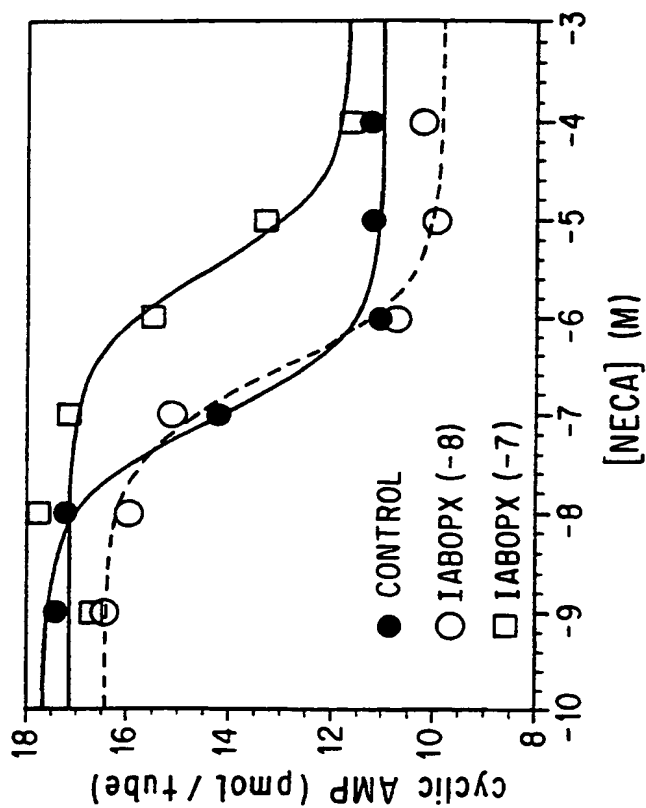


FIG. 13E

SUBSTITUTE SHEET (RULE 26)

21/ 31

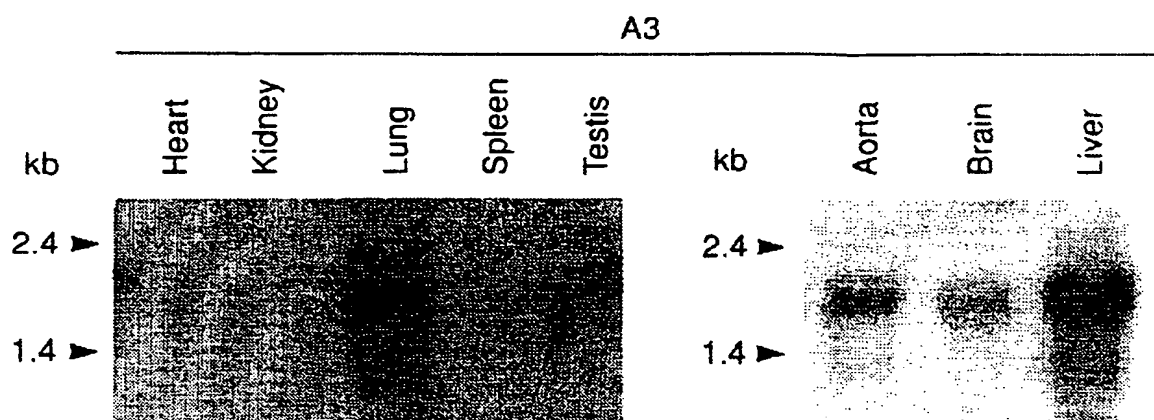


FIG.14A

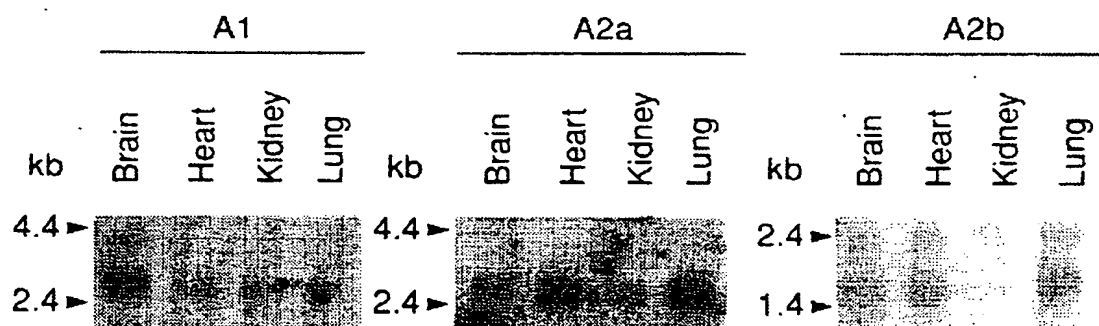


FIG.14B

22/31

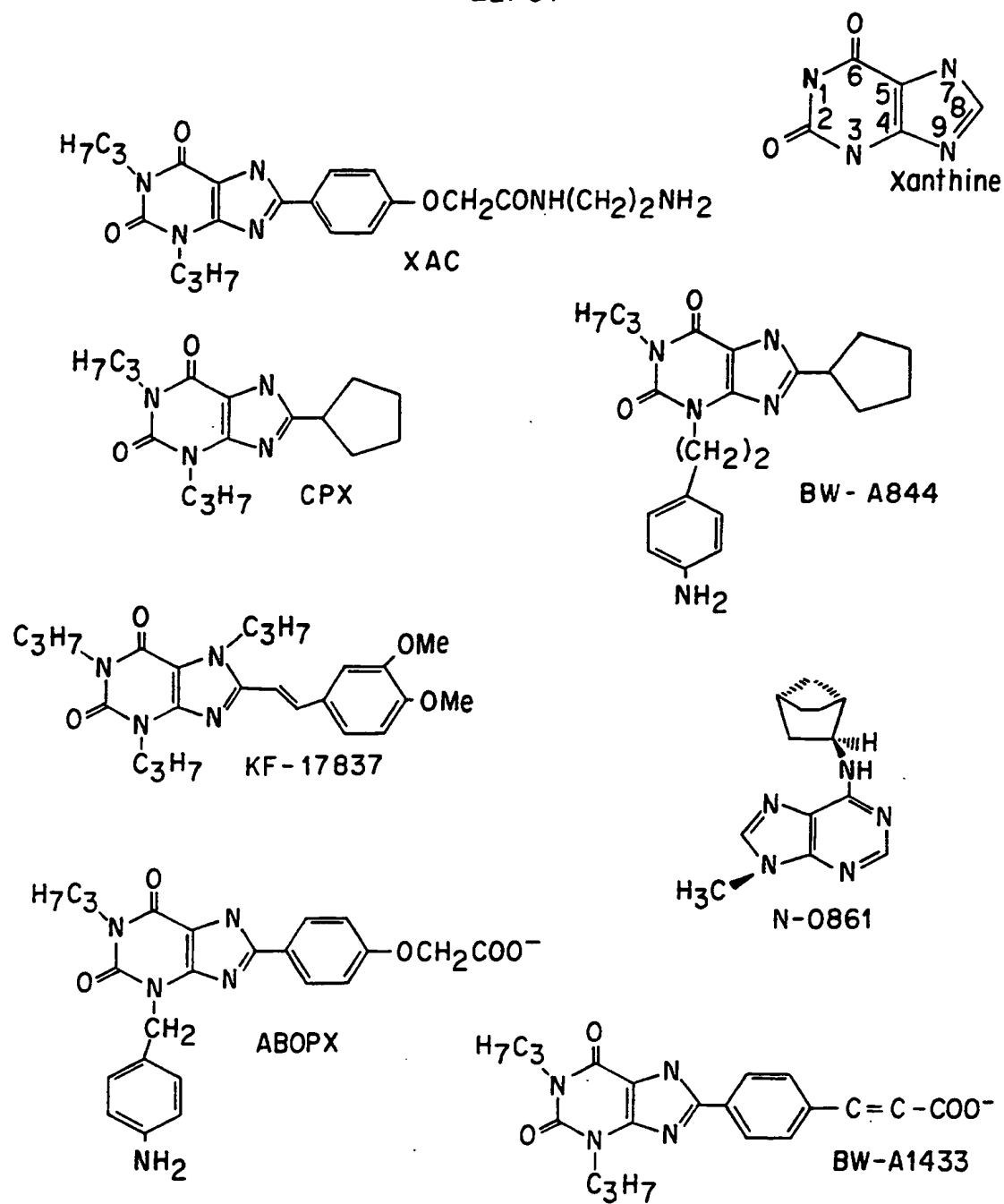


FIG. 15A

23/31

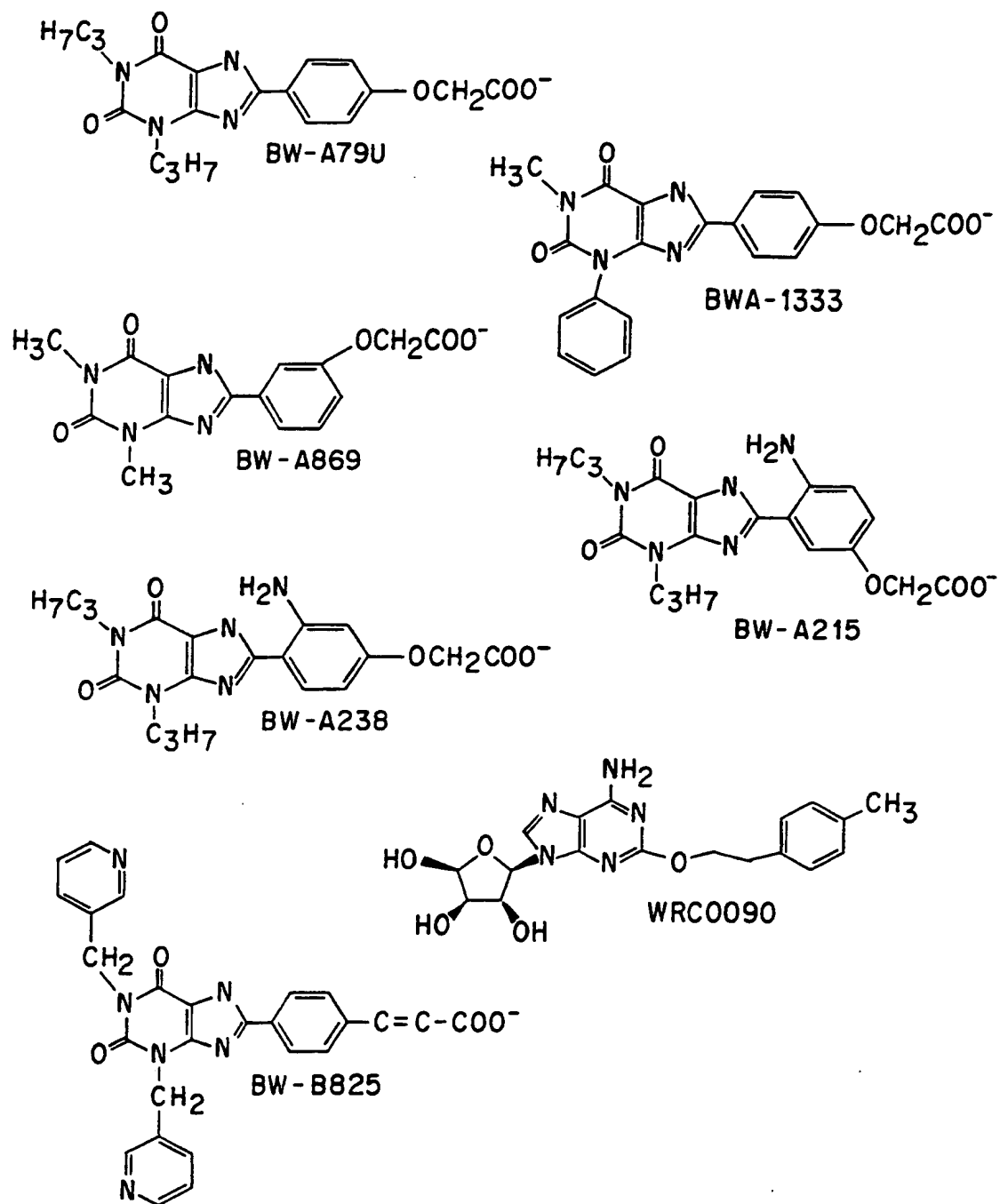


FIG. 15B

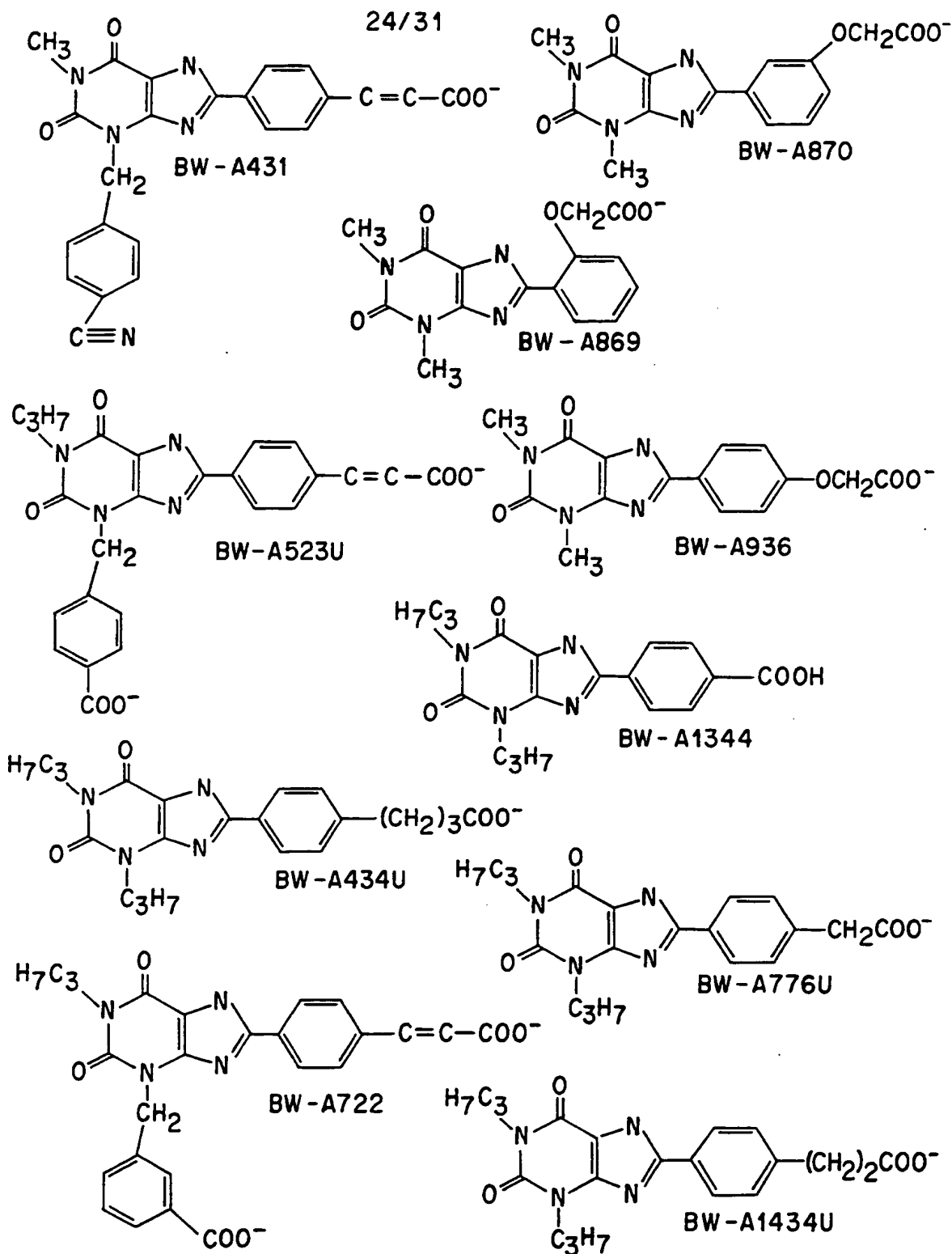


FIG. 15C

SUBSTITUTE SHEET (RULE 26)

25/31

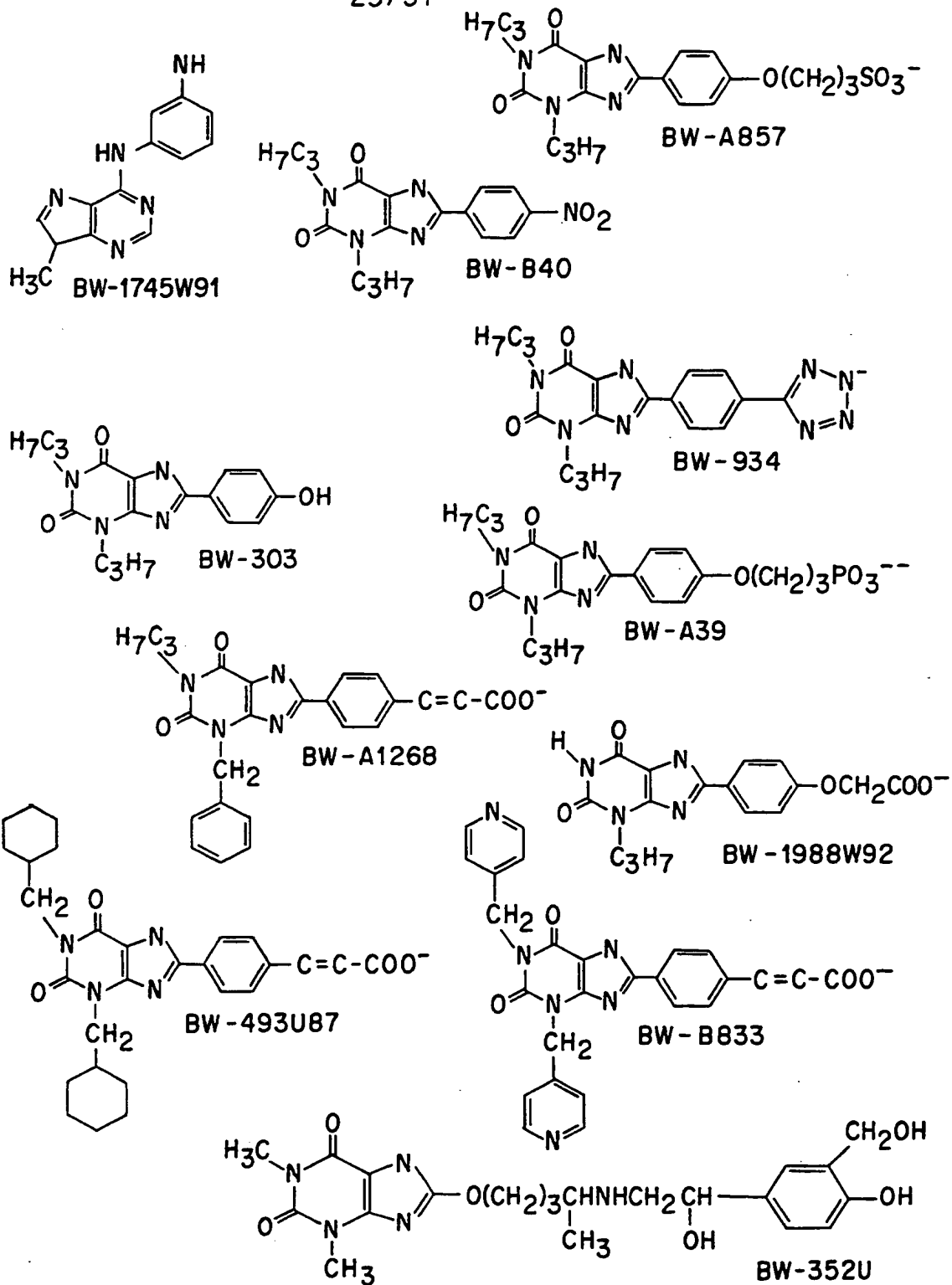


FIG. 15D

SUBSTITUTE SHEET (RULE 26)

26/31

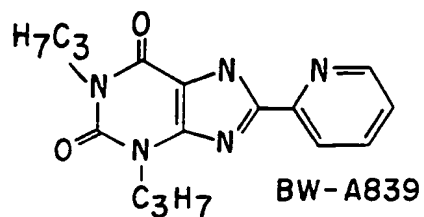
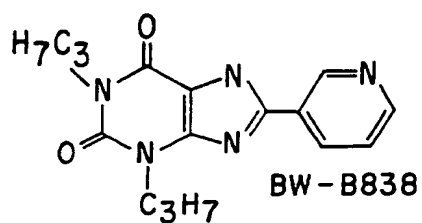
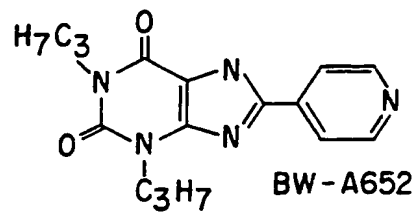
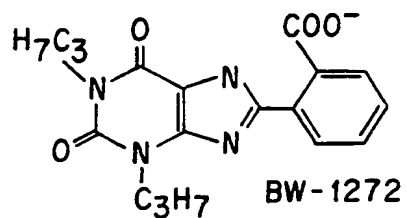


FIG. 15E

27/31



FIG. 16

28/31

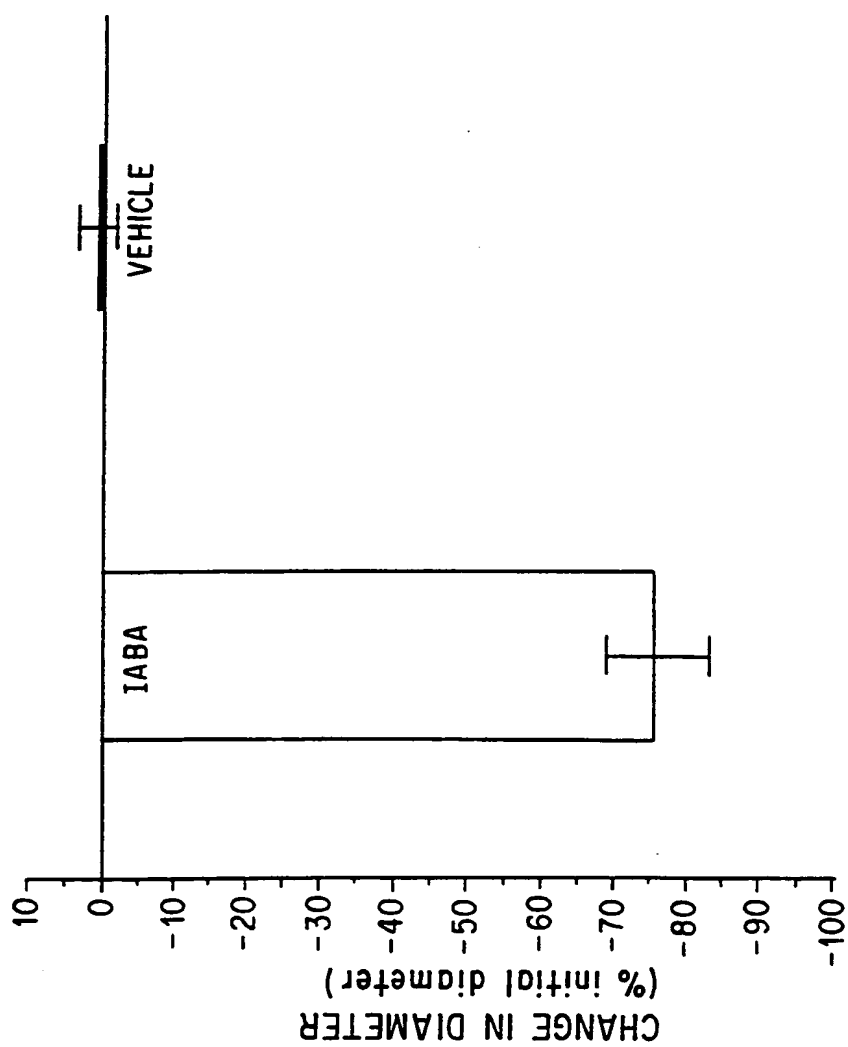


FIG. 17

29/31

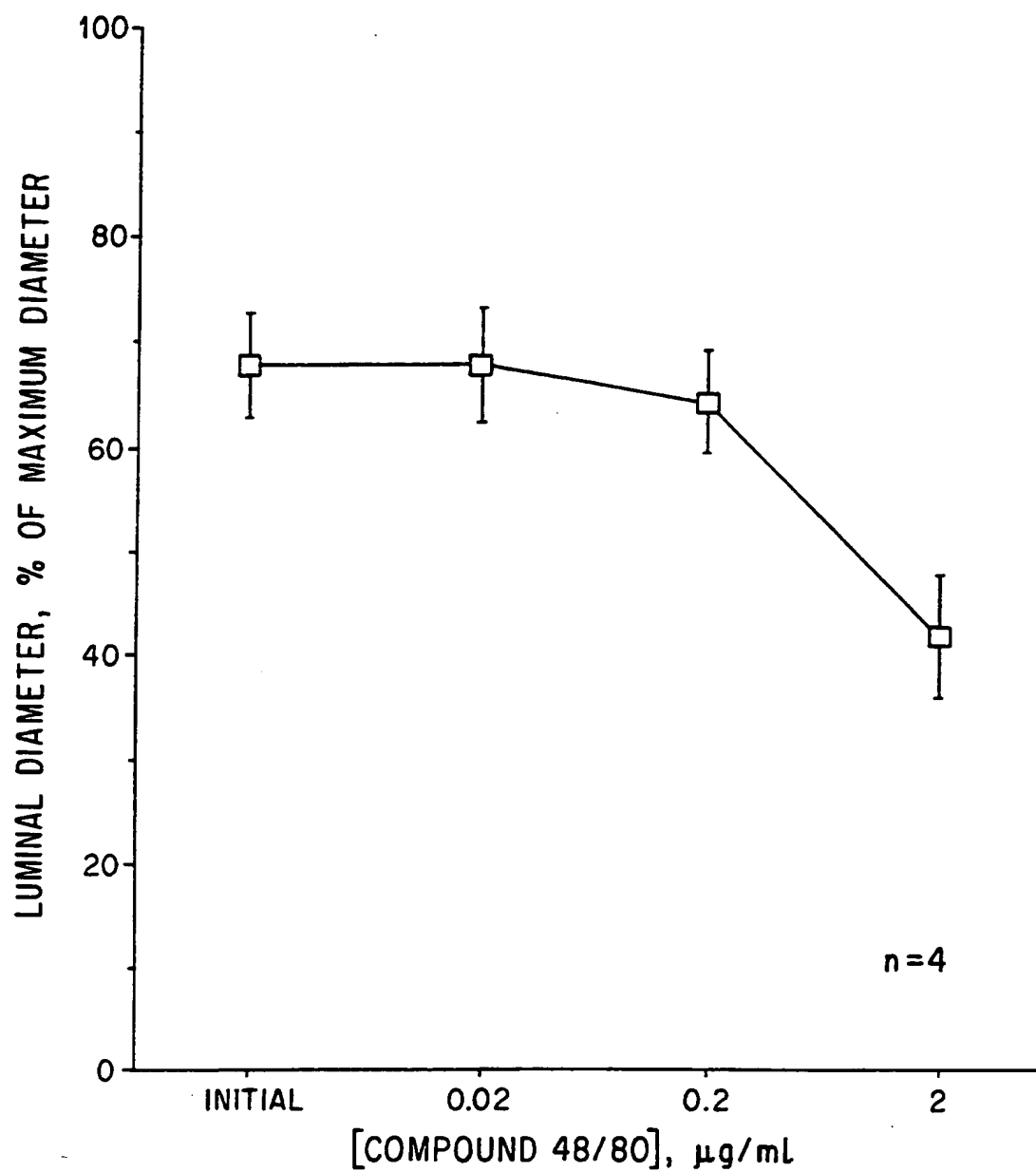


FIG. 18

30/31

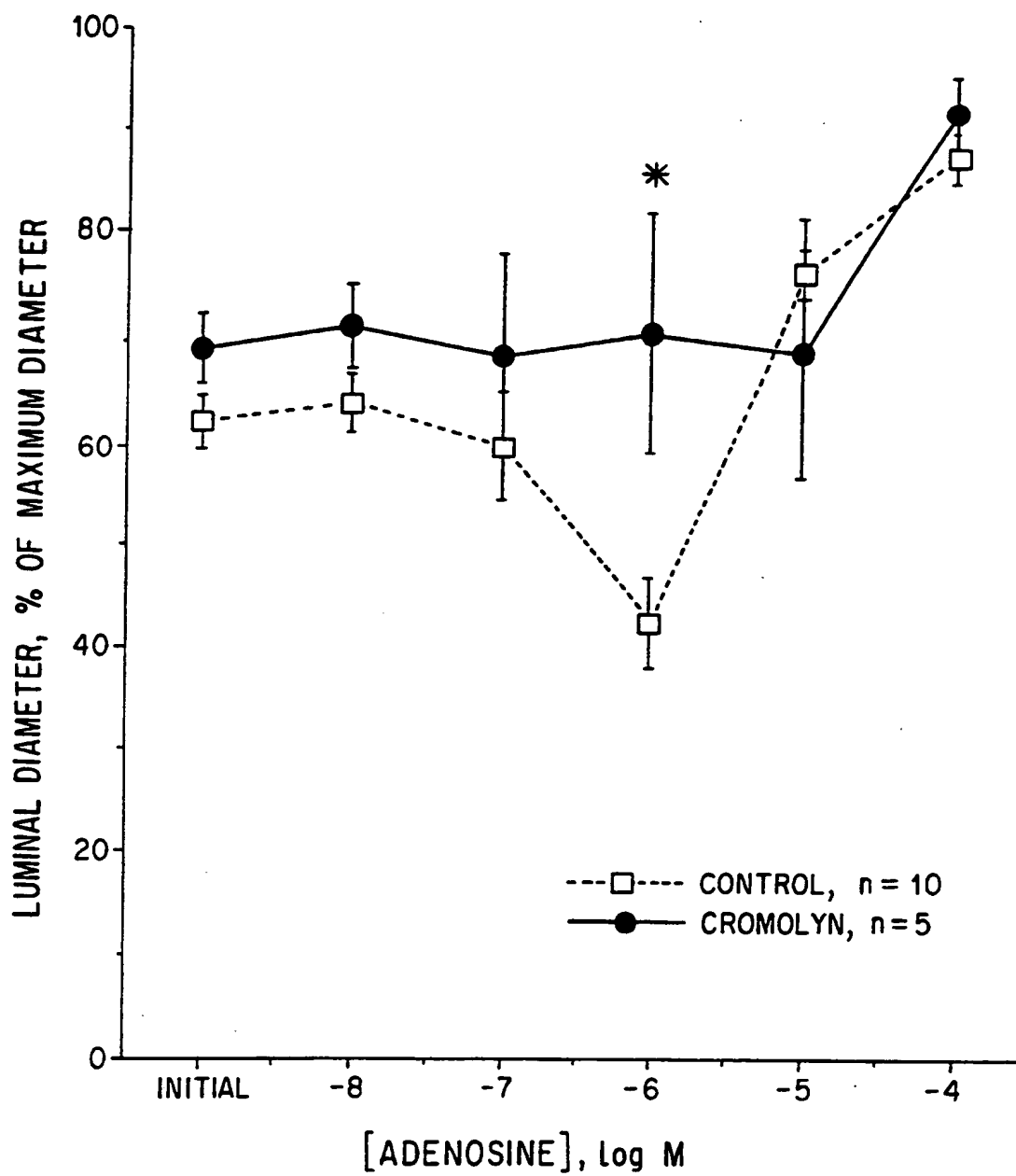


FIG. 19

31 / 31

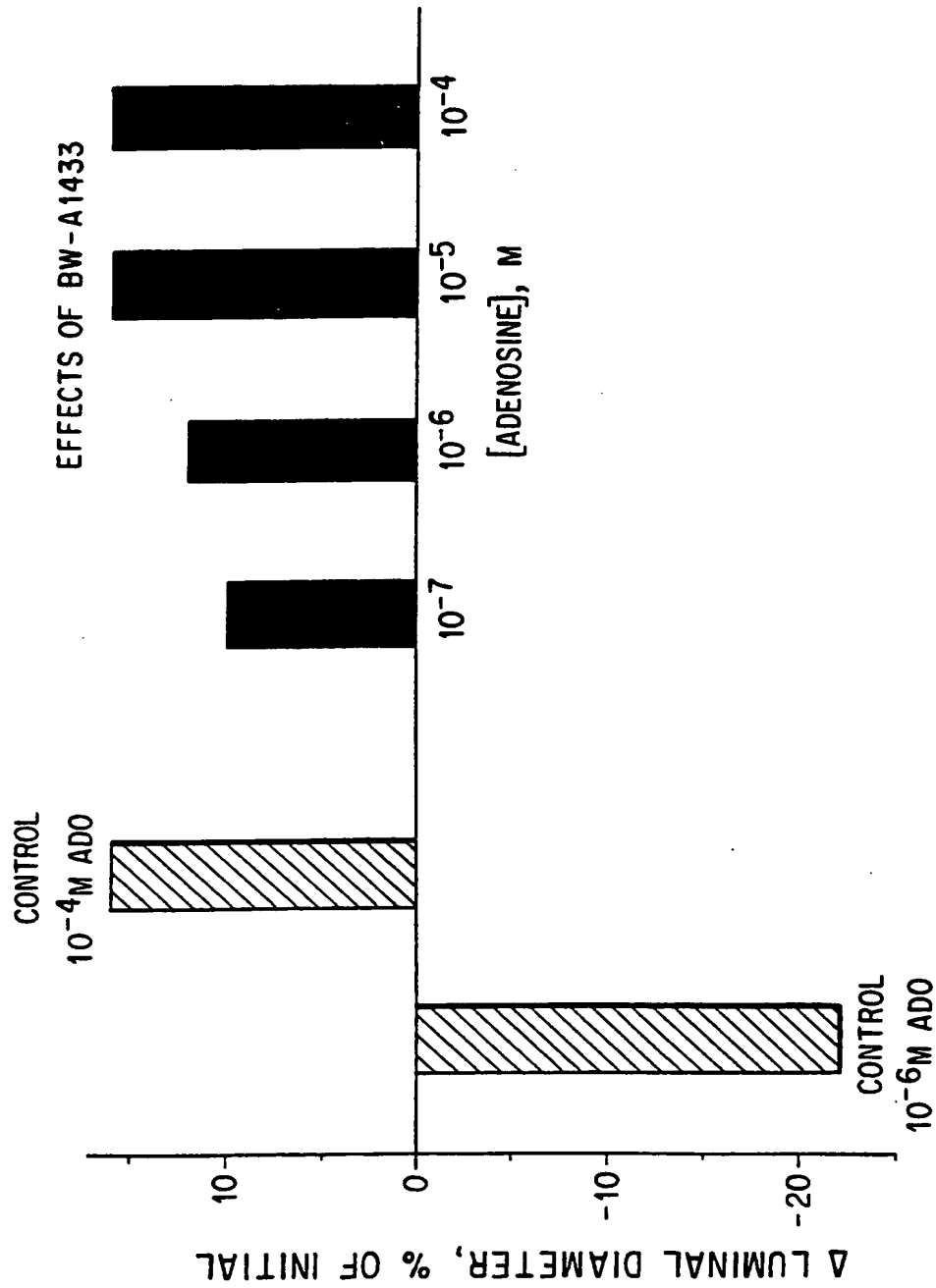


FIG. 20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12272

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/52

US CL :514/263, 265, 826, 929

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/263, 265, 826, 929

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, BIOSIS, MEDLINE ONLINE

SEARCH TERMS: ADENOSINE RECEPTOR, A3,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,879,296 (DALUGE ET AL.) 07 NOVEMBER 1989, column 1, lines 30-41 and 57-66 and claim 19.	1,2,12,16-22
X,P	US, A, 5,298,508 (JACOBSON ET AL.) 29 MARCH 1994, see entire document, specifically column 3, lines 20-25.	1-3,12,16-22
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 80, issued April 1983, Bruns et al., "Adenosine receptor binding: Structure-activity analysis generates extremely potent xanthine antagonists", pages 2077-2080, see entire document, specifically abstract.	2-3

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 31 JANUARY 1995	Date of mailing of the international search report 09 FEB 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARY CEBULAK Telephone No. (703) 308-1235

INTERNATIONAL SEARCH REPORT

 International application No.
 PCT/US94/12272

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DRUG DEVELOPMENT RESEARCH, Volume 30, issued 1993, Carruthers et al., "Hypotensive responses to the putative adenosine A3 receptor agonist N6-2-(4-aminophenyl)-ethyladenosine in the rat", pages 147-152, see entire document.	1,16-22
X	BRITISH JOURNAL OF PHARMACOLOGY, Volume 109, No. 1, issued Mar 1993, Fozard et al., "Adenosine A3 receptors mediate hypotension in the angiotensin II-supported circulation of the pithed rat", pages 3-5, see abstract.	16-22
X	JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 268, No. 23, issued 15 AUGUST 1993, Ramkumar et al., "The A3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells", pages 16887-16890, see entire document.	3,16-22
X	JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, Volume 258, No. 3, issued 1991, Ali et al., "Methylxanthines block antigen-induced responses in RBL-2H3 cells independently of the adenosine receptors or cyclic AMP: evidence for the inhibition of antigen binding to IgE", pages 954-962, see entire document, specifically abstract.	16-22
Y	INTERNATIONAL ARCHIVE OF ALLERGY AND APPLIED IMMUNOLOGY, Volume 98, issued 1982, Ott et al., "Effects of adenosine on histamine release from human lung fragments", pages 50-56, see entire document.	1-3,12,16-22
X	MOLECULAR PHARMACOLOGY, Volume 44, issued 20 SEPTEMBER 1993, Linden et al., "Molecular cloning and functional expression of a sheep A3 adenosine receptor with widespread tissue distribution", pages 524-532, see entire document.	1,16-22
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 89, issued August 1992, Zhou et al., "Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor", pages 7432-7436, see entire document, specifically page 7436.	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12272**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1, 2, 3, 12, and 16-22
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

- I. Claim 1, drawn to a method for achieving blockade of the vasoconstrictive response induced through adenosine activation of the A3 adenosine subtype.
- II. Claim 2, drawn to a method for treating or preventing myocardial ischemia, inflammation, brain arteriole diameter constriction, and the release of allergic mediators.
- III. Claims 3 and 12, drawn to a method for preventing or treating asthma, bronchoconstriction, allergic potentiation, inflammation or reperfusion injury in a human.
- IV. Claims 4 and 13, drawn to a method for preventing mast cell degranulation in a human.
- V. Claims 5-7, and 14-15, drawn to a method for achieving blockade of vascular constriction induced through activation of the A3 subtype of the adenosine receptor in a primate.
- VI. Claims 16-22, drawn to a method for achieving blockade of the A3 subtype of the adenosine receptor in a primate by contacting the receptor with a xanthine or xanthine derivative.
- VII. Claim 23, drawn to a method for treating an autoimmune disease.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the methods are related in so much as they utilize the same materials but each is drawn to a different therapeutic method, i.e., there is more than one inventive concept.